

Quantification and Understanding of the Fermentative Ability of Re-Pitched Yeast

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## **Abstract**

Brewer's collect yeast from one fermentation and re-pitch the cells into subsequent fermentations; this process is termed serial re-pitching. Deviations in the serial re-pitching process often impact the quality of the beer produced. Three variables within this process were investigated: the percentage of petite mutants in the pitched yeast, varying levels of dissolved oxygen in the wort and the length of yeast storage time between re-pitched fermentations. Results found that when petites in the culture increased from 3.7 % to 10.8 %, levels of esters and vicinal diketones were increased after the primary fermentation. The elevated concentrations of flavour compounds, however, would likely be undetectable to a consumer. When the yeast was subjected to 66 hour storage between fermentations, as opposed to 18 hour, the density attenuation rates during fermentation became less consistent. When investigating dissolved oxygen on serial re-pitching, the effect of low (3 mg/L) and high (16 mg/L) dissolved wort oxygen levels were not apparent on the flavours produced until the third fermentation; then, levels of propan-1-ol and 2,3-butanedione were increased with higher dissolved oxygen levels. Overall, these results could be used to help a brewer prioritize the controllable variables within the re-pitching process.

## **Dedication**

I dedicate this thesis to my family: my parents, Pauline and Ronnie Josey, my brother, David Josey, and my Nana, Dona Armstrong.

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## Abbreviations

%	percentage
°C	degrees Celsius
°P	degrees Plato
ABV	alcohol by volume
ADY	active dried yeast
ANOVA	analysis of variance
ASBC	American Society of Brewing Chemists
avg.	average
BC	before Christ
CCV	cylindroconical vessel
cm	centimetre
CO <sub>2</sub>	carbon dioxide
DHR	dihydrorhodamine
DMS	dimethyl sulphide
DNA	deoxyribonucleic acid
DO	dissolved oxygen
ECD	electron capture detector
EDTA	ethylenediaminetetraacetic acid
eq	equivalents
FAME	fatty acid methyl ester
FAN	free amino nitrogen
FID	flame ionisation detector
g	gram
GC	gas chromatography
hL	hectolitre
HOG	high-osmolarity glycerol
HPAE	high performance anion exchange
h	hour
IBU	International Bitterness Units
L	litre
M	Molarity
MCFA	medium chain fatty acid
mg	milligram
mL	millilitre
mm	millimetre
mRNA	messenger ribonucleic acid
MS	mass spectrophotometry
mtDNA	mitochondrial deoxyribonucleic acid
N	Normality
nm	nanometre
ns	not significant
O <sub>2</sub>	oxygen
psi	pounds per square inch
PVPP	polyvinylpolypyrrolidone

RD	respiratory deficient
ROS	reactive oxygen species
RS	respiratory sufficient
SD	standard deviation
SG	specific gravity
TCA	tricarboxylic acid
TPS	trehalose phosphate synthase
TTC	triphenyl tetrazolium chloride
U	units
VDK	vicinal diketone
VHG	very high gravity
wt	weight
μg	microgram
μL	microliter
μm	micrometre

## **Publications**

MacIntosh, A. J., Josey, M. and Speers, R. A. (2016) 'An examination of substrate and product kinetics during brewing fermentations', *Journal of the American Society of Brewing Chemists*, 74, pp. 250–257.

## Chapter 1 – Introduction

‘A journey of a thousand miles begins with a single step.’

-Confucius

Brewing produces a fermented beverage, beer. Beer brewing dates to as early as 3000 BC, and was first made in either Mesopotamia or Egypt (Nelson, 2005). However, there is evidence that suggests beer could date back to the Neolithic period, as early as 9000 BC (Hornsey, 2003; Meussdoerffer, 2009). In 2005, beer was the third most consumed beverage in the world after water and tea (Nelson, 2005). The single cellular eukaryotic microorganism yeast (Querol and Fleet, 2006) is responsible for taking the sugar solution, known as wort, and transforming it into beer. Yeast, particularly *Saccharomyces cerevisiae*, has been extensively researched (Uetz *et al.*, 2000; Lodolo *et al.*, 2008; Mathiasen and Lisby, 2014).

### 1.1 Yeast

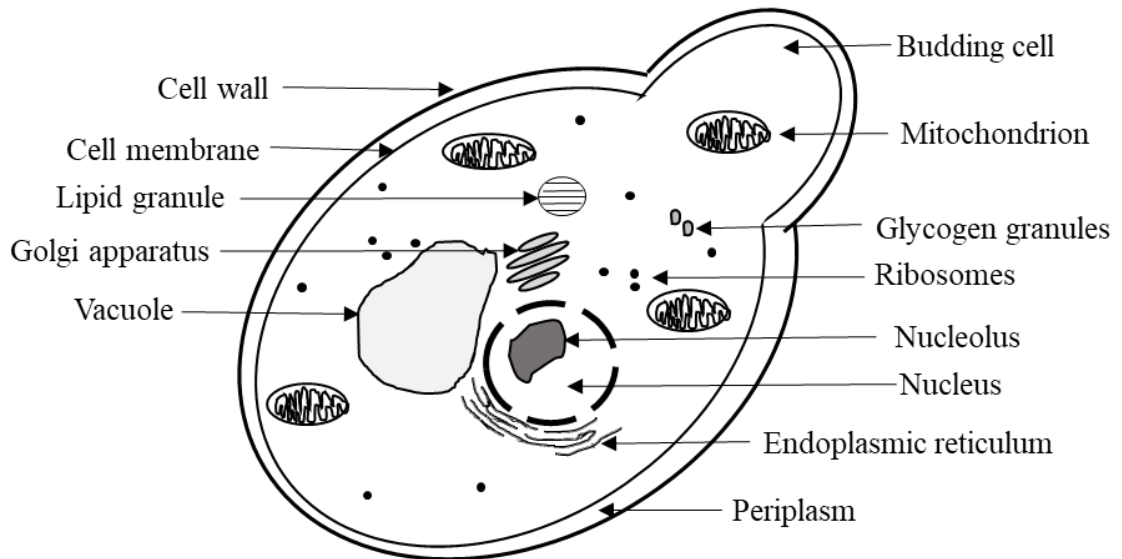
#### 1.1.1 Yeast Cell Cycle

Brewing yeast, most commonly *Saccharomyces cerevisiae*, undergoes asexual reproduction by budding. There are multiple stages of the cell division cycle: G1, S, G2, M, and cytokinesis. Before the cells start their division process, they rest in G1 phase. This stage is a gap period where the cell grows until it reaches an appropriate size, after which point the cell progresses through the rest of the cycle. At the end of G1, the cell will reach a point termed ‘Start’. If the cell does not have all essential nutrients to complete reproduction, it goes into a resting phase called G<sub>0</sub> (Briggs *et al.*, 2004). When the cell does pass Start, it enters the S phase where genome replication takes place and the bud that forms the daughter cell begins developing (Alberghina *et al.*, 2012). In G2, the bud continues growing. Finally, during mitosis (M phase), the duplicated copies of genome split into two, one for the daughter cell and the other for the mother cell (Boulton and Quain, 2001). The last stage of mitosis is cytokinesis where the mother and daughter cell are separated (Briggs *et al.*, 2004; Alberghina *et al.*, 2012).

#### 1.1.2 Cytology

The yeast cell contains many intracellular organelles enclosed by a plasma membrane and cell wall (Figure 1.1). The cell wall is the outermost layer of the yeast cell and is

anchored to the plasma membrane. The cell wall is made up of 90% carbohydrates and 10% proteins. When the bud of a new (daughter) cell separates from the mother cell, a bud scar is left on the mother's cell wall that is made of chitin. The daughter's cell wall is left with a birth scar, which is also made of chitin (Barton, 1950).



**Figure 1.1.** Typical budding yeast cell with its organelles denoted (adapted from Briggs *et al.*, 2004).

The space between the cell wall and the plasma membrane is the periplasm, and this contains secreted proteins from the cell (Venturini *et al.*, 1997). The plasma membrane is comprised of lipids that are arranged in a bilayer with integrated proteins. The lipids are phospholipids, and in small quantities sterols and unsaturated fatty acids (Van der Rest *et al.*, 1995). These lipids are crucial to the cell membrane health and overall viability of the yeast. Unsaturated fatty acids increase the membrane fluidity, which aids with cellular acclimatization (Rodríguez-Vargas *et al.*, 2007), and sterols play a role in endocytosis (Heese-Peck *et al.*, 2002). Additionally, the sterols aid in reducing the amount of ion “leakage” from the cells (Haines, 2001). The proteins that are integrated into the plasma membrane are involved with the uptake of nutrients, maintaining the proton motive force, and enzymes for cell wall synthesis.

The mitochondria are the location of oxidative phosphorylation and the electron transport chain that together generate ATP in the cell under de-repressing conditions. The mitochondria contain a small amount of its own DNA called mitochondrial DNA (mtDNA). The mtDNA encodes only 5% of proteins located in the mitochondria and the other 95% of mitochondrial proteins are encoded from the nuclear DNA. Mitochondria are involved with amino acid synthesis, 2,3-butanedione metabolism and

sterol biosynthesis (O'Connor-Cox *et al.*, 1996). The mitochondria are also the site for the tricarboxylic acid (TCA) cycle (James *et al.*, 2003). During fermentation when the cell is under anaerobic conditions, the majority of the mitochondria's respiratory activity is repressed (James *et al.*, 2003; Briggs *et al.*, 2004).

Other intracellular organelles are the nucleus and nucleolus, Golgi body, endoplasmic reticulum, and ribosomes. There are also vacuoles, glycogen granules and lipid granules inside the cell (Briggs *et al.*, 2004). The nucleus contains major cellular genetic material. The nucleolus is located within the nucleus and it contains several genes responsible for ribosomal biogenesis (Feldmann, 2012). The Golgi body is involved with processing proteins and modifying lipids. The endoplasmic reticulum is involved with modifying, and transporting proteins (Feldmann, 2012). Finally, the ribosomes are involved with protein synthesis (Kurata *et al.*, 2010).

### **1.1.3 *Petite Mutation***

The most common mutation found in brewing yeasts is the spontaneous deletion of either sections or the entire mtDNA (Šilhánková *et al.*, 1970b). These mutations are commonly called 'petites'. The name 'petite' is derived from the observation that mutated yeast colonies, when grown on agar plates, are abnormally smaller than a yeast colony without this mutation. Researchers have suggested that the negative effects of this mutation are slower growth, and inefficient sugar metabolism (Nagai *et al.*, 1961).

The mitochondria are important in aiding resistance to oxidative stress (Powell *et al.*, 2000a) and are also responsible for enzymes involved in the citric acid cycle, sterol biosynthesis, and amino acid synthesis (Visser *et al.*, 1994). One of the main defects of these cells is the inability to metabolize non-fermentable substrates such as glycerol and ethanol. Research has shown that petites lack cytochromes *a* and *b* and they still possess cytochrome *c* (O'Connor-Cox *et al.*, 1996).

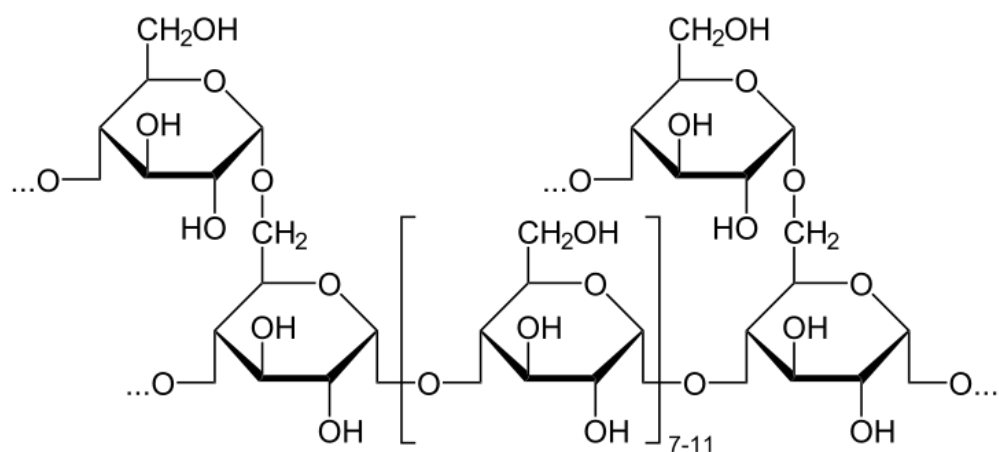
In brewing, one study reported an industrial lager yeast culture that had 50% of the yeasts mutated (Morrison and Suggett, 1983). However, the yeast culture in this study was stored in the fermenting vessel for over one week at 0 °C (Morrison and Suggett, 1983) and this practice does not represent the current practice in modern breweries. When serial repitching, many breweries have protocols for collecting the yeast immediately post fermentation, termed 'warm cropping' (Lawrence *et al.*, 2012). One study suggested that the presence of 2-4 % petites of the culture is acceptable (Donnelly and Hurley, 1996) and another study suggested that only when petite

mutations reach 10% of a population that their impact on fermentation becomes apparent (Van Zandycke *et al.*, 2002). Higher levels of petites present in fermentation have been associated with abnormally high levels of 2,3-butanedione (Ernandes *et al.* 1993). This may be connected with an alteration in branched amino acid synthesis by petites, specifically isoleucine and leucine which takes place in the mitochondria (Ryan and Kohlhaw, 1974). The compounds 2,3-butanedione and 2,3-pentanedione are by-products of the synthesis of those two amino acids (Section 1.3.6).

#### **1.1.4 Yeast Storage Carbohydrates**

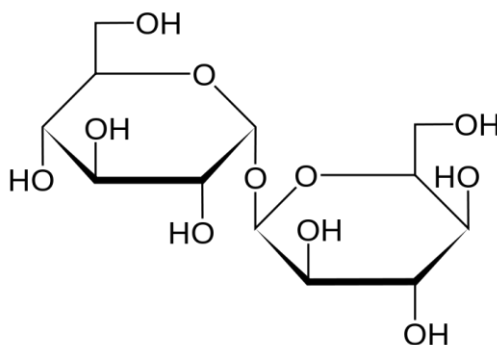
Glycogen (Figure 1.2) is a stored form of energy for the yeast cell (Quain *et al.*, 1981), comprising of branched glucose polymers. For glycogen synthesis, the protein glycogenin carries out initiation (François and Parrou, 2001). Next, elongation is undertaken by glycogen synthase and this enzyme catalyses an  $\alpha$ -(1 $\rightarrow$ 4)-glucosidic bond between two glucose molecules. Once the chains are formed, amylo-(1,4) $\rightarrow$ (1,6)-transglucosidase catalyzes an  $\alpha$ -(1 $\rightarrow$ 4)-glucosidic bond to form a branched point on the compound (François and Parrou, 2001). This is repeated until the compound is completed. One end of the compound has a reducing end and this is where the degradation of glycogen is initiated. Glycogen phosphorylase cleaves the glucose molecules from the chains (Hwang *et al.*, 1989).

At the beginning of fermentation, glycogen is the primary source of energy for the yeast. The energy from glycogen is utilized to synthesize unsaturated fatty acids and sterols for the cell (Bolat, 2008). Glycogen is synthesized towards the end of fermentation when fermentable sugars remain, however other nutrients (i.e. sulphur, nitrogen, or phosphorus) are limited (Boulton and Quain, 2001). At the end of fermentation when no fermentable sugars remain, glycogen is then utilized as an energy source (Bolat, 2008).



**Figure 1.2.** Section of the chemical compound, glycogen.

Trehalose (Figure 1.3) is a disaccharide that is made up of two glucose molecules. The key enzyme involved with the synthesis of trehalose is trehalose phosphate synthase (TPS). Trehalose also accumulates, like glycogen, when there are limited nutrients during fermentation. The degradation of trehalose, however, provides little energy (Bolat, 2008). This compound is involved in the cell's stress response for immediate survival (Martínez-Pastor *et al.*, 1996; Bolat, 2008). Trehalose is hypothesized to aid in thermo-tolerance (Elliot *et al.*, 1996) and also as an osmo-protectant (White *et al.*, 2003). Additionally, when the cell is exposed to ethanol, trehalose is synthesized to reduce the fluidity of the cell membrane (Gibson *et al.*, 2007).



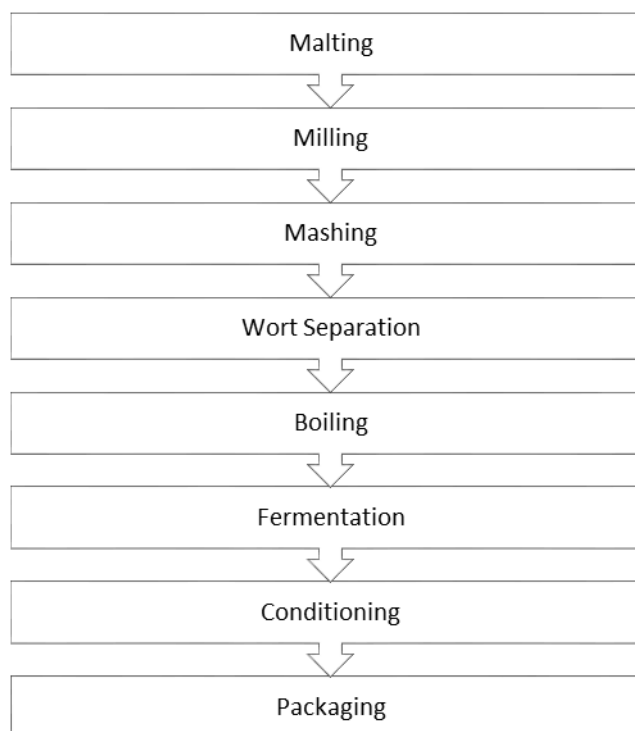
**Figure 1.3.** The chemical compound, trehalose.

## 1.2 Brewing Process

In its purest form, brewing uses four main ingredients: water, malted barley, hops, and yeast. Other added ingredients that are used to increase the carbohydrate content are termed adjuncts. Common adjuncts are rice, maize, or liquid sugars. The



production of beer is a multistep process that starts with the malting of barley and ends with packaging the final product (Figure 1.4).



**Figure 1.4.** Steps in the brewing process starting with malting and ending with packaging.

### **1.2.1 Malting and Milling**

Malting consists of three-unit operations: steeping, germination, and kilning (Briggs, 1998). During steeping, harvested barley grains are steeped in warm water, which allows the barley to absorb the water and start respiration. Typically, this step takes place between 12 – 15 °C (Brookes and Lovett, 1976). The grain absorbs water until it is 1.3 – 1.4 times its original size (Briggs *et al.*, 2004), during this time the moisture content of the barley increases to approximately 43 – 46 % (Shands and Dickson, 1953; Brookes and Lovett, 1976). When that happens, and once the chit (shoot) penetrates the husk of the barley, steeping is completed (Lewis and Young, 2001). During the germination period, the barley is agitated, and humid air at 15 – 22 °C is passed through the barley. Eventually the barley (embryo) produces hormones, such as gibberellins (Radley, 1967) that stimulate the production of enzymes. Once these enzymes are produced, germination is stopped by kilning. During kilning, the moisture of the barley is reduced and the enzymes are stabilized (Woffenden, H. M. 2002; Kim *et al.*, 1993). If germination continues then starch reserves that are necessary in mashing would be degraded. The enzymes produced through germination include

endoglucanases, endoproteases, and amylases (Bamforth, 2009), which are important in the mashing stage of brewing (Boulton and Quain, 2001) (Figure 1.1). Kilning stabilizes the malt and malt enzymes by reducing the moisture as low as 4 % (Shands and Dickson, 1953).

Before mashing, the malt is milled. The chosen mill depends on the process for wort separation. A roller mill is commonly chosen if a lauter tun will be used for wort separation. An appropriate ratio of full unbroken husk to fine powder is important when using a lauter tun and the roller mill is ideal for this because the rollers can be set a specific distance apart, thus controlling the fine or coarse grist to be milled. If a mash filter is used for wort separation, then a hammer mill is commonly used for milling. This will mill the grist into a fine powder like flour (Lewis and Young, 2001). Furthermore, the size of grain when milling impacts the extractability of the malt. A finer grind will yield a higher wort extract than a coarse grind (Mousia *et al.* 2004).

### **1.2.2 Mashing and Wort Separation**

Mashing is the process that converts starches in the mash vessel into fermentable sugars, such as maltose, maltotriose, and glucose. There are three main methods for mashing: infusion mashing, decoction mashing and double mashing. The first method, infusion mashing, is a single step mash. The grain is mixed with the water to achieve a temperature around 62 – 68 °C (Lewis and Young, 2001; Bilverstone *et al.*, 2015). This temperature allows gelatinisation to occur. For barely malt, gelatinisation normally occurs between 55 – 80 °C (Brandam *et al.*, 2003). Once gelatinisation occurs, the enzymes, such as  $\alpha$ -amylase, catabolize the starches into fermentable sugars.

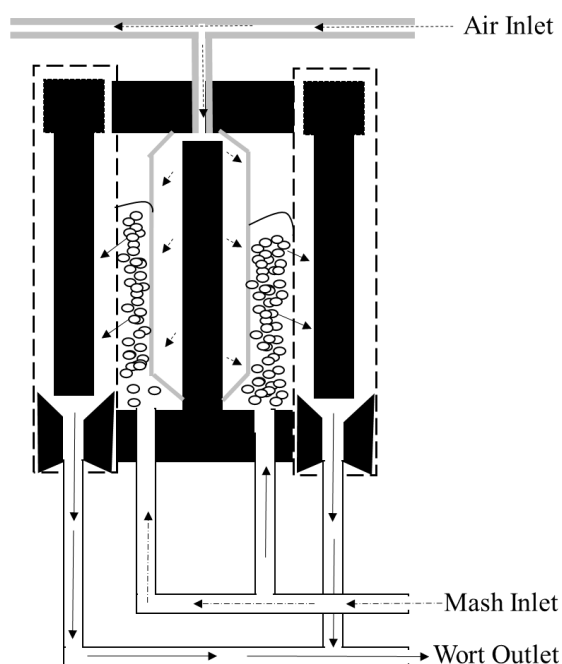
The second method for mashing is decoction mashing. The mash is subjected to two or three time periods, known as temperature rests, where the temperature is held constant. The first temperature rest is normally 35 – 40 °C (Lewis and Young, 2001). This temperature allows proteases to become active and break down protein. The temperature rest at this temperature is termed a protein rest. The second temperature achieved is typically 50 °C. This is the optimal temperature for  $\beta$ -amylase activity that converts starches to fermentable sugars. Lastly, the temperature is raised a third time by removing of a section of the mash, boiling, and re-adding it. The third temperature achieved is 65 °C, where  $\alpha$ -amylase action starts (Lewis and Young, 2001).

The last method is double mashing. Double mashing starts with two separate mashes: the main mash and the cereal mash. The cereal mash has adjuncts with low

enzymatic activities, such as rice. First, this mash is boiled because the gelatinisation of many adjuncts such as maize and rice starch, occurs at higher temperatures than barely malt (Thomas *et al.*, 1996; Bogdan and Kordialik-Bogacka, 2017). Once gelatinisation of the adjuncts occurs, the cereal mash is combined with the malt mash where starch conversion takes place via enzymes (Lewis and Young, 2001).

There are two main methods for solid and liquid separation resulting in the production of clarified wort: lautering or mash filtration. Lautering involves gravity separation in which wort is clarified by passage through a bed of grains held within a vessel containing a perforated false bottom. After mashing is complete, the mash is transferred to a lauter tun. The grain sits on a false bottom that is held just above the bottom of the tank forming a bed. The plate has thin (0.5 – 1 mm wide) slots that allow the wort to pass through (Lewis and Young, 2001) and the bed acts as a filter to retrieve wort that is clear of spent grain. At the beginning of wort separation, the collected wort is recirculated back into the lauter tun because it will contain small particle matter that slipped through the grain bed and false bottom. When the retrieved wort is clear, the wort is transferred into the kettle for wort boiling.

Alternatively, a mash filter can be used. A mash filter is made up of plates with frames or a frame supported with a mesh cloth. In between those plates, there are other plates that contain a deeper frame. The mash is pumped into the frame with the deeper cavity. With pressure applied, the sweet wort filters through the cloth into the smaller cavity. The sweet wort is transferred from the smaller cavity into the kettle for boiling (Figure 1.5).



**Figure 1.5.** Diagram of one chamber in a mash filter starting to fill with the grain and wort from the mash (adapted from Briggs *et al.*, 2004). The mash inlet, wort outlet, and airline are labelled.

### 1.2.3 Wort Boiling and Hop Additions

When the spent grain is separated from the wort, the wort is boiled in the kettle. Denk *et al.* (2000) describe the many reasons for boiling the wort:

1. “Inactivation of malt enzymes,
2. Sterilization of the wort,
3. Extraction and isomerization of compounds derived from hops,
4. Coagulation of protein material in the wort,
5. Formation of protein/polyphenol complexes,
6. Formation of flavour and colour complexes,
7. Formation of reducing substances to give the wort reducing potential, which is thought to protect the wort from oxidation later in the process,
8. Fall of wort pH,
9. Concentration of wort gravity through evaporation of water,
10. Evaporation of volatile compounds in wort derived from mashing,
11. Evaporation of volatile compounds in wort derived from hops.”

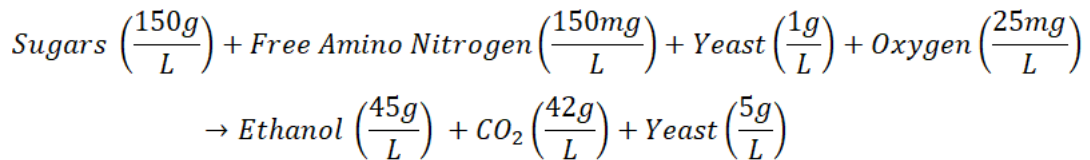
Hops may be added at any point during the boil. Hops contain  $\alpha$ -acids and  $\beta$ -acids that isomerize to iso- $\alpha$ -acids or iso- $\beta$ -acids with heat (Briggs *et al.*, 2004). When hops are added early during the boil, higher degrees of isomerization happen, which

lead to more bitter beers. The hop bitter acids may lower the pH of the boil (Briggs *et al.*, 2004). A few examples of volatile compounds that evaporate during boiling are dimethyl sulphide,  $\beta$ -damascenone, benzaldehyde, and methional (De Schutter *et al.*, 2008; Scheuren *et al.*, 2016).

At the end of wort boiling, a precipitate is formed of proteins (Delcour and Vanhamel, 1988). These are removed using a whirlpool before transferring the wort to the fermenter. Additionally, the wort is cooled by passing the hot wort through a plate and frame heat exchanger. Often zinc losses are observed during this step, which could be detrimental to fermentation (Kühbeck *et al.*, 2006).

#### 1.2.4 Fermentation

To start fermentation, yeast is inoculated in wort at a pitching rate typically between  $15 - 20 \times 10^6$  cells/mL. The yeast metabolizes the fermentable sugars and free amino nitrogen (FAN) in the wort and produces ethanol and carbon dioxide (Equation 1.1) (Boulton and Quain, 2001). In small quantities, yeast utilize zinc, magnesium, and calcium during fermentation (Silverstone *et al.*, 2015). Additionally, small amounts of esters, higher alcohols, vicinal diketones and sulphurs are produced (Dickinson, 2008; He *et al.*, 2014; Pires *et al.*, 2014). Specific details on the fermentation biochemistry will be discussed later (Section 1.3).



(Equation 1.1)

The course of primary fermentation can take three to ten days. The time depends on various factors of the fermentation such as the original density of the wort, temperature, dissolved oxygen content, the pitching rate, and yeast strain (Nagodawithana *et al.*, 1974). Normal wort densities are  $<16$  °P, ‘high gravity wort’ densities are  $16 - 18$  °P and ‘very high gravity wort’ densities are  $20 - 25$  °P (Gibson, 2011). Oxygen is necessary at the beginning of fermentation for unsaturated fatty acid and sterol biosynthesis allowing cell growth (Kirsop, 1974). Glucose is the first monosaccharide to be taken up by the yeast in fermentation. The presence of glucose in a medium causes a yeast repression pathway called catabolite repression pathway. This pathway inhibits multiple genes including those for maltose and maltotriose uptake, and respiration (Kayikci and Nielsen, 2015).

The sugar consumption profile of yeast from wort follows an “s” shaped sigmoidal regression (MacIntosh *et al.*, 2016). In the beginning, there is a lag period where the yeast does not metabolize many fermentable sugars in the medium. Then there is an exponential decrease as the sugars are metabolized at an increasing rate. Half way through the fermentation, the rate of sugar uptake reaches a point where it starts to slow down. Once the sugar consumption has stopped, the attenuation plateaus (MacIntosh *et al.*, 2016).

As the fermentation reaches an end, the yeasts flocculate. Flocculation is the phenomenon that yeast cells adhere to each other and form aggregates (Stewart and Russell, 1981). Calcium is necessary for flocculation to take place (Speers, 2016). Flocculent types of yeast possess FLO genes (Halme *et al.*, 2004). The genes encode flocculins that are located on the cell wall and they bind to mannans, on neighbouring yeast cells (Halme *et al.*, 2004). Also, environmental conditions, such as ethanol concentration, pH, or temperature of the medium, may impact on the cell’s ability to flocculate (Jin and Speers, 2000).

There are two largely used types of yeast in traditional fermentations: lager and ale yeast. Lager yeast are often referred to under the names of *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, and in the past, *Saccharomyces cerevisiae* (Bokulich and Bamforth, 2013; Wendland, 2014). Lager yeasts made an appearance in the 15<sup>th</sup> century (Meussdoerffer, 2009). *Saccharomyces pastorianus* evolved from the natural hybridization of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (Libkind *et al.*, 2011). These yeasts ferment at lower temperatures than ale yeasts (Gibson *et al.*, 2013), and the fermentations typically take place in closed stainless steel cylindroconical vessels (CCV). These are ideally insulated and fitted with cooling jackets allowing fermentation temperature to be controlled at 7 – 15 °C (Pires and Brányik, 2015). As fermentation nears completion, the yeasts flocculate and settle to the bottom of the tank. There is a sample port at the bottom of the tank at the cone where the yeast may be collected if needed for re-pitching.

Ale yeasts, typically *Saccharomyces cerevisiae* (Bokulich and Bamforth, 2013), and are commonly known as ‘top fermenters’. Historically these fermentations took place in square open fermenters, some of which are still in use today. Ale yeast flocs trap CO<sub>2</sub> during flocculation, which makes them buoyant so they will rise to the top of the fermenter forming a Krausen layer (Saerens *et al.*, 2008).

After the primary fermentation, the beer contains harsh flavour characteristics, and high vicinal diketone (VDK) levels. A diacetyl rest typically takes place to adjust the unwanted flavours. The two major VDKs produced during fermentation are diacetyl (2,3-butanedione) and 2,3-pentanedione (White and Wainwright, 1975; Krogerus and Gibson, 2013). These two compounds are responsible for a buttery and/or butterscotch property to the beer (Pires and Brányik, 2015). These compounds are perceived as off-flavours and undesirable in most beer styles. For conditioning, the temperature is reduced to around 4 °C, and approximately 1 – 4 million cells (Briggs *et al.*, 2004) are left in suspension. These cells absorb 2,3-butanedione and convert it to 2,3-butanediol. Diol compounds have higher flavour thresholds, which are undetectable in the beer (Pires and Brányik, 2015).

### **1.2.5 Conditioning**

During conditioning the beer is clarified, which improves colloidal stability (Aron and Shellhammer, 2010). This process typically takes place around -1 °C. The haze may be removed by a number of methods: adding finings, enzymes, and/or by the natural polymerisation of polyphenols (Bamforth, 1999). Additionally, polyvinylpolypyrrolidone (PVPP) and protein removers such as silica gels may be added to the beer at this point to reduce haze (Bamforth, 1999). PVPP removes polyphenols, which over time forms haze in packaged beers (Aron and Shellhammer, 2010).

The beer may also be sterile filtered or pasteurised to produce a beer that is relatively microbiologically stable (Portno, 1968). Finally, the beer may be carbonated by streaming CO<sub>2</sub> through the beer. This process is called forced carbonation. Equipment to set up this process is expensive and smaller craft breweries may choose to bottle condition. This involves adding priming sugar to the beer before it is bottled, causing residual yeast to ferment, naturally increasing the CO<sub>2</sub> levels.

### **1.2.6 Yeast Handling**

During or prior to conditioning, the yeast is collected by either warm or cold cropping. For warm cropping, the yeast is collected at the end of the primary fermentation, at that time the beer is at a higher temperature. Alternatively, when cold cropping, the yeast sediments in the CCV during conditioning and is collected at the end. Yeast autolysis may occur when cold cropping. Autolysis is the irreversible self-digestion of the cell using intracellular enzymes that ends in cell death (Babayan and Bezrukov, 1985; Alexandre and Guilloux-Benatier, 2006). Autolysis takes place during

stationary phase growth when challenging environmental conditions are present in the cone (Alexandre and Guilloux-Benatier, 2006). Yeast autolysis causes unpleasant astringent and bitter flavours to the beer. When 5 % of the yeast present in a population undergo autolysis, beer quality becomes a problem (Xu *et al.*, 2014).

The collected yeast, from either warm or cold cropping, is still viable and therefore may be used for another fermentation. Between fermentations, the yeast is stored between 3 and 4 °C (Somani *et al.*, 2012). During yeast storage, the yeast is typically stored unagitated under beer or water (McCaig and Bendiak, 1985). The impact that agitation and different temperatures during storage have on yeast health will be discussed later (Section 7.1). Overall, it is crucial to maintain the yeast's fermentative ability and physiological condition (O'Connor-Cox, 1998).

Some breweries choose to acid wash the yeast before pitching the stored yeast into the next fermentation. Acid washing is rinsing the yeast culture with phosphoric acid, or sometimes sulphuric acid or acidified ammonium persulphate, to eliminate bacterial contamination (Simpson and Hammond, 1989). If yeast is acid washed, this step will take place immediately prior to yeast pitching. During this process, the acid is below 5 °C and constant mixing takes place. This step should not take more than two hours (Simpson and Hammond, 1989).

### **1.2.7 Packaging**

After conditioning, beer can be transferred to kegs, casks, cans, or bottles for sale. At this stage, understanding the impact of ageing on beer quality is important. The quality of the beer will be impacted with time, and it is difficult to control once the beer leaves the brewery. Once packaged, some of the reactions that take place are the degradation of hop bitter acids, and the formation of aldehydes. Aldehydes that can form during storage of packaged beers, contribute to the 'staling' off flavour (Vanderhaegen *et al.*, 2007). By reducing the oxygen in contact with packaged beer, some reactions may be impeded. Bottled beer with air in the headspace causes drastic production of free radicals, specifically H<sub>2</sub>O<sub>2</sub>, in the beer in 4 hours when incubated at 60 °C (Kaneda *et al.*, 1989). This particular free radical is generally believed to be associated with increased levels of aldehydes, associated with staling, and degradation of the hop component isohumulones (Kaneda *et al.*, 1989).



## **1.3 Fermentation Biochemistry**

### **1.3.1 Carbohydrate Assimilation**

Carbohydrates are the preferred source of energy for the yeast cell and are metabolized by glycolysis. Glycolysis is the pathway in the cell that metabolizes glucose and leads to the synthesis of ethanol in yeast (Barnett, 2003; Gibson *et al.*, 2008a). The majority of ale and lager yeast metabolize glucose, sucrose, fructose, maltose, galactose, raffinose, maltotriose, and sometimes trehalose (Phillips, 1955; Boulton and Quain, 2001). Additionally, lager yeast can metabolize melibiose (Bendiak *et al.*, 1994). The uptake of sugars is highly ordered (Stewart and Russell, 2009): glucose, fructose, and sucrose are first metabolized simultaneously, then maltose, and last maltotriose (Phillips, 1955; Briggs *et al.*, 2004). For the uptake of sucrose, the sugar is first hydrolysed by invertase in the periplasmic space into its constituent units of glucose and fructose, which can then enter the glycolytic pathway (Lagunas, 1993). However, in a fermenting medium, overlap exists between the individual sugar uptake orders (MacIntosh *et al.*, 2016).

### **1.3.2 Mineral Utilization**

Along with the sugar consumption, the yeast also uptake minerals. These are necessary for cellular enzymes and the essential ions include boron, calcium, cobalt, iron, potassium, magnesium, manganese, nickel, and zinc. The presence of metals, such as iron, copper, zinc and manganese, play a role in the function of haem-proteins, cytochromes, redox pigments, and enzyme cofactors (Deželak *et al.*, 2015a). One particularly important ion is zinc. Zinc is important for the activity of alcohol dehydrogenase and other enzymes (Magonet *et al.*, 1992). Without this metal, a fermentation may become “stuck” meaning the yeast stop metabolizing the fermentable carbohydrates, leaving the final gravity higher than normal (De Nicola and Walker, 2011). This problem also exists in wine fermentations (Bisson, 1999).

These metals are necessary for successful fermentation, however high concentrations, particularly with heavy metal ions, may be toxic and exert stress the cells (Briggs *et al.*, 2004). During fermentation, the metals are accumulated by the yeast and the yeast also release metal ions back into the fermenting medium. Yeast quality (Mochaba *et al.*, 1996) and serial re-pitching (Aleksander *et al.*, 2009; Deželak *et al.*, 2015a) are two factors that affect the yeast’s uptake and release patterns of the minerals.

### 1.3.3 Vitamins

Most yeast requires small quantities of vitamins during fermentation (Graham *et al.*, 1970). Some of these vitamins may include pantothenic acid, nicotinic acid, biotin, riboflavin, inositol, pyridoxin, and/or thiamine (Graham *et al.*, 1970). Brewing wort compositions naturally consist of many of these vitamins (MacWilliam, 1968).

### 1.3.4 Nitrogen Utilization

Yeast need nitrogen for growth during fermentation. FAN is the preferred nitrogen source by yeast. FAN includes all available individual amino acids, dipeptides and tripeptides (Lekkas *et al.*, 2005). Of the nitrogenous contents in lager-type wort, there are approximately 20 % protein, 30 – 40 % polypeptides, 30 – 40 % amino acids, and 10 % nucleotides (Ingledew, 1975). For normal density fermentations, a minimum FAN concentration between 140 – 150 mg/L is recommended (Jones and Rainbow, 1966). Amino acids are assimilated from the wort in a particular order (Table 1.1). Class A amino acids are quickly assimilated in the beginning of fermentation in a linear pattern (O'Connor-Cox and Ingledew, 1989). Class B amino acids are absorbed by yeast at a slower rate than the absorption of Class A amino acids. Class C amino acids are only absorbed once Class A amino acids are completely removed from the medium (O'Connor-Cox and Ingledew, 1989). Class D contains proline, which is not normally assimilated by yeast during fermentation, however, this may be strain specific as a previously published study demonstrated that there was a reduction of proline in the wort during one fermentation (Gibson *et al.*, 2009).

**Table 1.1.** Classification of amino acids in groups based on assimilation by yeast from the wort during fermentation (Pierce, 1987).

Class A	Class B	Class C	Class D
Arginine	Histidine	Alanine	Proline
Asparagine	Isoleucine	Ammonia	
Aspartate	Leucine	Glycine	
Glutamate	Methionine	Phenylalanine	
Glutamine	Valine	Tryptophan	
Lysine		Tyrosine	
Serine			
Threonine			

### 1.3.5 Lipid Uptake

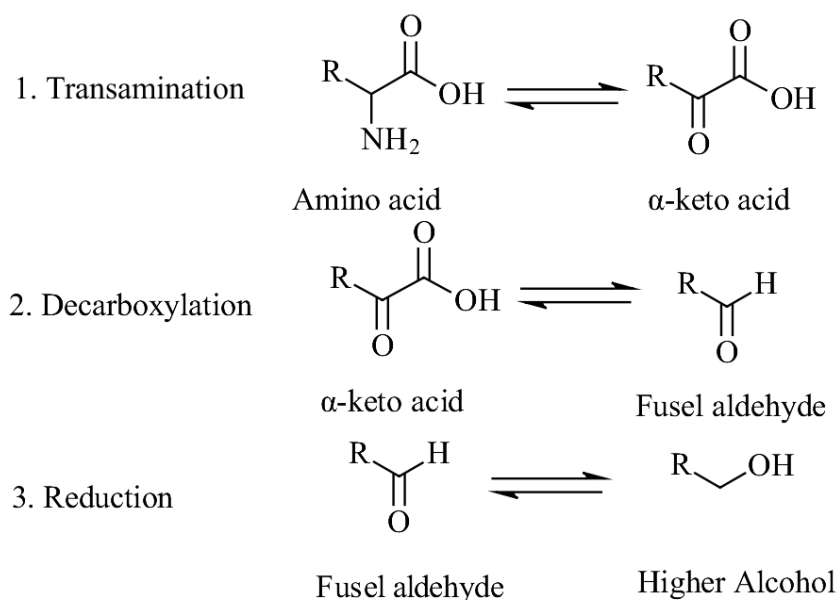
Yeast either synthesizes lipids, or takes them up from the wort to maintain normal levels of lipids. For sterol and unsaturated fatty acid synthesis, oxygen is

necessary (Boulton and Quain, 2001; Lodolo *et al.*, 2008). During fermentation, lipid synthesis takes place during the lag period when oxygen is present. Fatty acid synthesis has a high energy requirement (Martin *et al.*, 2007). If not synthesised *de novo*, the cell can take lipids in from an external supply or by endogenous lipid turnover (Briggs *et al.*, 2004; Tehlivets *et al.*, 2007). The hydrophobic nature of fatty acids is critical in the structure of the plasma membrane and compartmentalization *in vivo* (Tehlivets *et al.*, 2007). The unsaturated fatty acids are utilized in the yeast cell membrane that aid in ability to withstand osmotic stress and ethanol levels (Piper *et al.*, 1994).

### **1.3.6 By-Products of Fermentation**

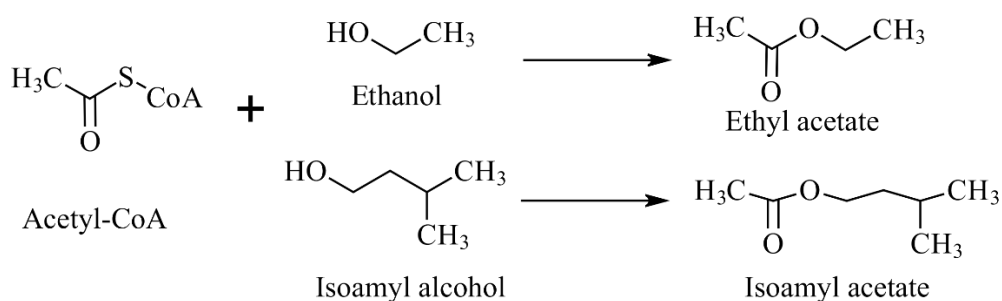
Vicinal diketones, esters, higher alcohols, and sulphurs are minor by-products from the fermentation, but largely contribute to the flavour and aroma profile of the beer. Many esters and higher alcohols possess desirable compounds, whereas vicinal diketones and sulphurs are commonly viewed as off-flavours (Krogerus and Gibson, 2013; Pires *et al.*, 2014).

Higher alcohols (Table 1.2) play an important role in beer quality. Common higher alcohols in brewing are propan-1-ol, isobutanol, 2-methylbutanol and 3-methylbutanol, and 2 phenyl ethanol (Pires *et al.*, 2014). These compounds contribute solvent and sweet flavours to the beer. Higher alcohols are also precursors in ester synthesis. Higher alcohols are synthesized by either the Ehrlich Pathway starting with an amino acid, or by *de novo* with the wort carbohydrates (Pires *et al.*, 2014). During fermentation both pathways are utilized, but in the early stages of fermentation, the Ehrlich Pathway (Figure 1.6) is the predominant pathway for higher alcohol synthesis by utilizing wort amino acids (Schulthess and Ettlinger, 1978). The Ehrlich Pathway has three steps: transamination, decarboxylation, and reduction (Pires *et al.*, 2014).

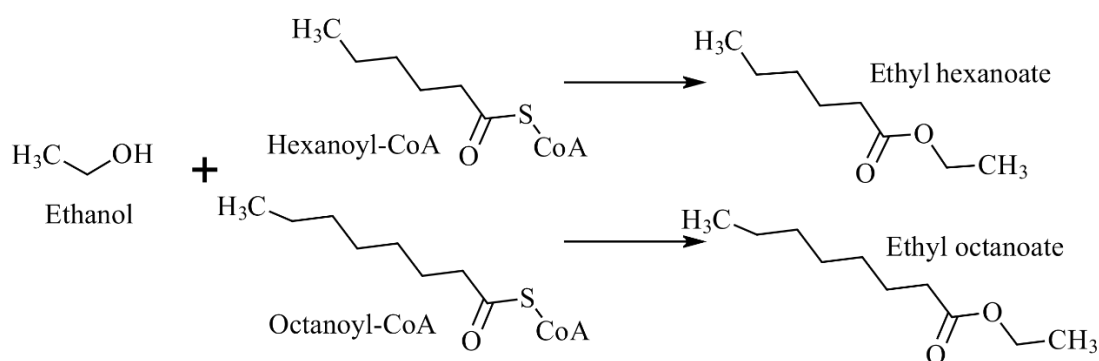


**Figure 1.6.** The three steps in the Ehrlich pathway to form a higher alcohol (adapted from Pires and Brányik, 2015).

Esters also contribute to the flavour and aroma profile of beer, and are often associated with pleasant fruity flavours and aromas (Table 1.2). Although esters are present in very small quantities, these compounds have very low odour thresholds (Suomalainen and Nykänen, 1966; Engan, 1972; Saison *et al.*, 2009). In high quantities, these compounds become unpleasant (Pires *et al.*, 2014). The two main groups of esters are medium-chain fatty acid (MCFA) ethyl esters and acetate esters (Saerens *et al.*, 2010). Acetate esters are formed by an alcohol and acetyl CoA, and MCFA ethyl esters by ethanol and fatty acyl CoA (See Figure 1.7 and 1.8) (Pires *et al.*, 2014).



**Figure 1.7.** Acetate ester synthesis (adapted from Pires and Brányik, 2015).



**Figure 1.8.** MCFA ethyl ester synthesis (adapted from Pires and Brányik, 2015).

Vicinal Diketones (VDKs) are largely unwanted compounds in the beer as they give off a buttery or toffee-like flavour (Table 1.2). The two most common VDKs found in beer are 2,3-butanedione (diacetyl) and 2,3-pentanedione. Diacetyl is generally the focus of most research, as it has a much lower flavour threshold than 2,3-pentanedione (Table 1.2). When yeast biosynthesizes valine and isoleucine,  $\alpha$ -acetoxy acids are excreted from the cell. In the medium, the acids convert to 2,3-butanedione or 2,3-pentanedione by spontaneous oxidative carboxylation (Hardwick, 1994; Boulton and Quain, 2001). This takes place during fermentation. However, from the middle to late fermentation, the yeast cells reabsorb the converted VDKs and metabolize diacetyl to acetoin and then 2,3-butanediol (Krogerus and Gibson, 2013). As well, 2,3-pentanedione is metabolized to 3-hydroxy-2-pentanone and then 2,3-pentenediol (Krogerus and Gibson, 2013). Sometimes, higher than normal levels of VDKs in the beer may be associated with contamination by *Lactobacillus* or *Pediococcus* species (Christensen and Pederson, 1958; Boulton and Quain, 2001).

**Table 1.2.** Common flavour compounds produced as by-products from the brewing fermentation with their flavour threshold, common concentrations found in beer, and general impression.

Compound	Threshold (mg/L)	Typical Ranges (mg/L)	Aroma Impression	Source
<i>Acetate Esters</i>				
Ethyl acetate	25-30	8 – 32	Fruity, solvent	(Pires and Brányik, 2015)
Isoamyl acetate	1.2-2	0.3 – 3.8	Banana	(Pires and Brányik, 2015)
Isobutyl acetate	0.5	N/A	N/A	(Engan, 1972)
Ethyl butyrate	0.3	N/A	Pineapple	(Aroxa, n.d.)
<i>MCFA ethyl esters</i>				
Ethyl hexanoate	0.2-0.23	0.05 – 0.21	Apple, fruity	(Pires and Brányik, 2015)
Ethyl octanoate	0.9-1.0	0.04 – 0.53	Apple, aniseed	(Pires and Brányik, 2015)
<i>Higher Alcohols</i>				
Propan-1-ol	600	4 – 17	Alcohol, sweet	(Pires and Brányik, 2015)
Isobutanol	100	4 – 57	Solvent	(Pires and Brányik, 2015)
2-methyl butanol	65	N/A	Alcohol, medicinal	(Olaniran <i>et al.</i> , 2017)
3-methyl butanol	50-70	25 – 123	Alcoholic, banana	(Olaniran <i>et al.</i> , 2017; Pires and Brányik, 2015)
<i>VDKs</i>				
2,3-butanedione	0.1-0.15	0.02 – 0.07	Sweet, buttery	(Pires and Brányik, 2015)
2,3-pentanedione	0.9-1.0	0.01 – 0.02	Buttery, toffee-like	(Pires and Brányik, 2015)

## 1.4 Serial Re-Pitching

### 1.4.1 Introduction

Yeast is commonly recycled between fermentations within breweries. Recycling yeast involves using the same yeast culture in subsequent fermentations until no longer fit for use (Kobayashi *et al.*, 2007; Kordialik-Bogacka and Diowks, 2013, Miller *et al.*, 2013). This cycle is called serial re-pitching.

The brewing yeast cycle starts with yeast propagation. Yeast propagation normally starts with the inoculation of a stock culture of yeast into a growth medium with constant aeration on a laboratory scale. The biomass produced from that growth

stage is re-inoculated into a fresh growth medium. Sequential cultures are generated and the scale of the propagation is increased until sufficient biomass is produced for a production scale fermentation (Briggs *et al.*, 2004). Propagation can take multiple days depending on the mass of yeast necessary, which in turn depends on fermentation volume. In one brewery, to propagate yeast for an 800-hL fermentation, a five-step yeast propagation was necessary, a process that could take weeks (Maule, 1979). Propagation takes less time when utilizing Active Dried Yeast (ADY) because the use of ADY circumvents the need for this propagation process. ADY should be properly rehydrated and then pitched into the fermenters (Jenkins *et al.*, 2011). Once the correct mass is obtained, the yeast is pitched into the first fermentation. With serial re-pitching, this is often called generation 0, or crop 1. The second fermentation that uses the same yeast culture is called generation 1, or crop 2 and increases accordingly as the yeast is re-pitched. It is established common practice that beer from the first fermentation is blended with re-pitched fermentations because the beer has poor quality compared to the beer produced from re-pitched fermentations. When the first fermentation ends, the yeast flocculates and these cells are collected and stored in beer. The yeast is stored around 4 °C until pitched into the subsequent fermentation. Prior to re-pitching, some breweries will choose to acid wash the yeast, however other breweries decide to omit this step. Ale and lager yeast are both resistant to low pH during acid washing, however, a combination of some conditions, such as high temperature or high ethanol can be detrimental to the yeast health (Simpson and Hammond, 1989). Acid washing is controversial (Simpson and Hammond, 1989; Smart and Wilcocks, 1995). The practice helps reduce bacterial contamination, however at the same time may lower the yeast health (Cunningham and Stewart, 2000).

After yeast storage and if chosen, acid washing, the yeast is pitched by either volume or weight into the proceeding fermentation. Post fermentation the yeast is collected once flocculated, stored and after cell counting, and re-pitched repeating the cycle. The number of times a yeast crop is reused is very often limited to anywhere from two to twenty times (Table 1.3) depending on several factors, such as original gravity, yeast strain, and company policy.

**Table 1.3.** Published studies with reports of the number of fermentations that one yeast culture was typically recycled in the industry.

Ale or lager fermentations	Number of fermentations	Reference
Lager	6	(Bühligen <i>et al.</i> , 2014)
Lager	6	(Bühligen <i>et al.</i> , 2014)
Lager	17	(Bühligen <i>et al.</i> , 2014)
Lager	13	(Speers and Stokes, 2009)
Lager	10	(Speers <i>et al.</i> , 2003)
Lager	135	(Powell and Diacetis, 2007)
Lager	20	(Bühligen <i>et al.</i> , 2013)
Ale	30	(Smart and Whisker, 1996)
Ale	5	(Kobi <i>et al.</i> , 2004)
Ale	98	(Powell and Diacetis, 2007)

When the cultures of yeast are re-cropped numerous times, they are repeatedly exposed to fluctuations in environment conditions (Table 1.4).

Oxidative stress and osmotic stress may occur during propagation and the beginning of fermentation in the serial re-pitching process. Initially cells are exposed to oxidative stress when oxygen is present. With the presence of oxygen, the cells utilize  $O_2$  and reactive oxygen species (ROS) are formed such as  $O_2^-$ ,  $H_2O_2$ , or  $OH^-$  (Beckman and Ames, 1998). ROS can cause damage to nucleic acids, proteins, and lipids in the cell (Ikner and Shiozaki, 2005). Some strains of yeast may have a higher tolerance to oxidative stress than others (González-Párraga *et al.*, 2008). The yeast may experience osmotic stress when concentrations of solutes are high. This happens in the beginning of propagation and fermentation in VHG wort. White and colleagues (2003) demonstrated that viability of yeast cells when exposed to high solute concentration was highly strain dependent. This study investigated how the viability of three ale strains and four lager strains were affected by incubation in 12 % sorbitol and 30 % sorbitol (White, Kennedy and Smart, 2003). High osmotic stress can cause several changes to the cell: cell shrinking, loss of turgor, and long term causes such as growth arrest. The high-osmolarity glycerol (HOG) pathway is the yeast response to this stress (Yang *et al.*, 2006).



**Table 1.4.** Different conditions and stresses exposed to the yeast during propagation, fermentation, and storage (Gibson *et al.*, 2007).

Time during stage	Propagation	Fermentation	Storage
Beginning	Osmotic stress Oxidative stress	Oxidative stress Osmotic stress Anaerobic shift	Cold shock Ethanol toxicity Nutrient limitation
Throughout	Oxidative stress		Cold shock Ethanol toxicity Nutrient limitation
End	Oxidative stress	Ethanol stress Nutritional limitation Cold shock	Cold shock Ethanol toxicity Nutrient limitation

Oxidative and osmotic stresses decline as both the dissolved oxygen levels in the wort and carbohydrate concentrations decrease. Towards the end of the lag period, the yeast transitions to anaerobic fermentation. As the yeast ferments, the ethanol concentration increases and the nutrients decrease. Ethanol toxicity and nutrient limitations become stresses that the cell may encounter nearing the end of fermentation. Each yeast strain has a specific concentration of ethanol tolerance before ethanol toxicity becomes apparent in the cell (Casey and Ingledew, 1986; You *et al.*, 2003). There are two periods during serial re-pitching that yeasts are exposed to sub-optimal nutrient levels: at the end of fermentation and during yeast storage between fermentations.

Another dynamic environmental condition that yeast must adapt to during serial re-pitching is the temperature. During propagation, the temperature may be held at 21 °C (Webber *et al.*, 1952) or higher (Boulton & Quain, 2001). The temperature during fermentation ranges from 6 to 15 °C or 18 to 25 °C depending on a lager fermentation or ale fermentation respectively (Gibson *et al.*, 2007). As yeast flocculates post fermentation, the temperatures in the cone can vary from 2 – 11 °C. Controlling the temperature in the center of the cone is difficult to maintain. The yeast continues to metabolize, producing heat. Once fermentation is completed, the yeast is collected and again stored at a low temperature of 1 to 4 °C. Temperature changes can have several effects to the cell (Table 1.5).

**Table 1.5.** Physiological changes of yeast during temperature changes.

Temperature Change (Increased or decreased)	Physiological Change
Increased	Increase in membrane permeability (Marza, Camougrand and Manon, 2002)
Increased	Increase in heat shock proteins (hsp) (Piper, 1995)
Increased	Reduction in sterol levels (Piper, 1995)
Increased	Reduction of unsaturated lipids (Piper, 1995)
Increased	Lower intracellular pH (Piper, 1995)
Decreased	Reduction in cell fluidity (Piper, 1995)

The last major environmental factor that the yeast cells must adapt to during serial re-pitching is pH. This declines rapidly at the beginning of fermentation and continues to decline at a slower rate as the fermentation progresses (Coote and Kirsop, 1976) starting from pH 5.5 and decreasing to approximately 4.1 as CO<sub>2</sub> is produced during the fermentation (Gibson *et al.*, 2007). Changes in pH between fermentations can have an impact on the concentrations of flavour compounds produced during fermentation such as diacetyl (Haukeli and Lie, 1978) and dimethyl sulphide (Anness and Bamforth, 1982).

#### **1.4.2 Impact on Fermentation Characteristics**

##### *Density Attenuation*

In one study, ten successive serial re-pitched fermentations were monitored for seven different brands of industrially fermented beers (Speers *et al.*, 2003). The crop numbers showed no significant ( $p > 0.05$ ) changes in maximum fermentation rate, the midpoint of the fermentation or the final gravity values. As these parameters had demonstrated no changes for the ten successive fermentations, this suggested that the yeast cultures could be re-pitched past ten times (Speers *et al.*, 2003). Later, Speers and Stokes (2009) monitored serial re-pitched fermentations up to crop number 13. This study found that no significant differences existed ( $p > 0.05$ ) between the crop number and the density at 48 h, the time to reach final density, the Apparent Degree of Fermentation (ADF), and the final pH (Speers and Stokes, 2009). Additionally, no flavour changes were noted in brewery productions tastings (A. Speers, *pers. comm.*, August 12<sup>th</sup>, 2017).

Laboratory scale fermentations found similar results related to density attenuation. Two serial re-pitched fermentations showed that the sugar uptake and metabolism had no significant differences between the two fermentations (Miller *et al.*,

2013). The same conclusion was found with a study on serial re-pitched 400 mL fermentations (Kordialik-Bogacka and Diowksz, 2013). The study monitored the final gravities for eight serial re-pitched fermentations in 10 °P wort and ten serial re-pitched fermentations in 15 °P wort (Kordialik-Bogacka and Diowksz, 2013). Both of the serial re-pitched fermentations were performed with two lager yeast strains and found no significant differences ( $p > 0.05$ ) between the final gravities in the re-pitched series.

Another study monitored density by gravity of serial re-pitched fermentations comparing ADY to wet yeast. The study showed that freshly propagated wet yeast fermented at a quicker rate than the ADY. The second fermentation (G1) showed a shorter lag period for both dried and wet yeast (Powell and Fischborn, 2010).

In contrast to common industrial practices and beliefs, the literature showed that crop number had no impact on attenuation. However, most studies did not analyse the attenuation throughout the entire fermentation and some of these studies were performed on a small-scale.

#### *Volatile Compounds*

The control of flavour compounds produced during fermentation are important as they affect the quality of the final product. For this reason, flavour compounds are commonly monitored when investigating the effects of crop number on fermentation and the final product.

Kobayashi and colleagues (2007) found increased levels of isoamyl alcohol produced post fermentation when the crop number increased. The concentrations of isoamyl alcohol reported were above the 50 – 65 mg/L detection threshold (Kobayashi *et al.*, 2007), speculating that the differences between crop numbers may have been detectible. Another study investigated serial re-pitching and levels of amyl alcohol (both isoamyl alcohol and 2-methyl butanol together). This study found that amyl alcohol levels produced post fermentation increased until crop five, and then decreased until crop seven. Additionally, ethyl acetate levels present post fermentation were increased with higher crop numbers (Quilter *et al.*, 2003).

Powell and Fischborn (2010) completed an industrial ingestion of re-pitched ADY and wet yeast from crop 1 (G0) to crop 5 (G4). Higher alcohols, esters, and dimethyl sulfide (DMS) were monitored. Conversely from the previous two studies mentioned, a decrease in isoamyl alcohols levels was observed from G0 and G4. There were differences in flavour volatiles produced between G0 and G4, and a sensory difference (triangle) test done between these showed the crops could be distinguished.

Interestingly, this study showed that the ADY and wet yeast utilized showed no differences between the flavours produced (Powell and Fischborn, 2010).

A laboratory scale study analysed ten successive fermentations with 10 °P wort and eight successive fermentation with 15 °P wort using two lager strains of *Saccharomyces pastorianus* in 400 mL of wort (Kordialik-Bogacka and Diowks, 2013). This study investigated esters and higher alcohols and concluded no general rise in ester and higher alcohol formations was observed with increased generation number (Kordialik-Bogacka and Diowks, 2013).

Many studies investigated changes in higher alcohols and esters (Moonjai *et al.*, 2003; Quilter *et al.*, 2003; Kobayashi *et al.*, 2007; Deželak *et al.*, 2015b). The final VDK levels with respect to the yeast crop number are also important for the quality of the beer. Cutaia and colleagues (2009) found that the levels of VDKs present post fermentation were unchanged between the crop numbers.

A tall tube fermentation study examined flavour changes in beer fermented with freshly propagated yeast and with yeast after three successive fermentations (Pickerell, *et al.*, 1991). This study considered acetaldehyde, diacetyl, and sulphur compounds and compared to the glycogen content. The conclusion of this study determined that when there were low levels of glycogen in the pitching yeast, there was poor yeast growth, a slower attenuation, and lower rates of uptake for Class B and Class C amino acids (Pickerell *et al.*, 1991).

The nature of the low flavour thresholds for esters, vicinal diketones and higher alcohols indicate that slight changes in the levels produced may have adverse effects of beer quality. The studies presented demonstrate that the effects of the production of these flavour compounds on serial re-pitching are not universal.

### **1.4.3 Impact on Yeast**

Instead of considering the beer produced, many studies have investigated the effects that serial re-pitching has on the yeast cells. Understanding the effect that the serial re-pitching process has on the cells, may indicate when the culture is no longer fit for fermentation.

#### *Minerals Uptake*

Yeast uptake minerals during fermentation. Metal ions, such as calcium, magnesium, and zinc, are essential enzyme co-factors and these affect cellular growth (Saltukoglu and Slaughter, 1983; Boulton and Quain, 2001). One study investigated various ion contents in the beer throughout four serial re-pitched fermentations

(Aleksander *et al.*, 2009). The study found that the yeast in the first fermentation accumulated higher levels of calcium, magnesium, and zinc when compared to the proceeding fermentations (Aleksander *et al.*, 2009). The study looked at the ion content both in the yeast cell mass and in the fermenting wort. Results showed that during the oxygenated propagation stage, the yeast biomass had three fold more magnesium than at the end of the anaerobic fermentation process (Aleksander *et al.*, 2009).

Another study examined iron, copper, manganese and zinc levels in fermenting wort over 11 successive fermentations (Deželak *et al.*, 2015a). In this study, quinoa wort, buckwheat wort, and barley wort were investigated. In the first barley wort fermentation, the zinc levels in the wort decreased in the first 24 hours. However, in successive fermentations, the zinc levels increased, meaning the yeast released zinc into the fermentation. The trend of yeast releasing zinc during successive fermentations was only observed with barley wort and was not observed with buckwheat or quinoa wort (Deželak *et al.*, 2015a).

#### *Nitrogen assimilation*

A study investigating beer polypeptides in three serial re-pitched industrial fermentations showed that the first fermentation was significantly different and the second and third fermentations were not significantly different from the others (Vieira *et al.*, 2012). Another study investigated the polypeptides present in the foaming during fermentation with successive fermentations. Their results demonstrated that the first and second fermentations experienced the most foaming and the highest concentrations of polypeptides in the foam were observed with these fermentations (Kordialik-Bogacka and Ambroziak, 2007). This study also observed lower volumes of foaming were present in the subsequent fermentations (Kordialik-Bogacka and Ambroziak, 2007). These authors, therefore, hypothesized that extended serial re-pitched reduces foaming during fermentation (Kordialik-Bogacka and Ambroziak, 2007).

Another study investigated the nitrogen compound assimilation more closely by monitoring FAN and amino acid uptake by yeast during a fermentation with propagated yeast and the subsequent re-pitched fermentation (Miller *et al.*, 2013). These authors found that concentrations of histidine, isoleucine, leucine, valine and all amino acids from Group C were higher in the beer fermented with re-pitched yeast compared to the freshly propagated yeast. Additionally, proline, was not assimilated from either fermentation (Miller *et al.*, 2013). The authors proposed that cells will preserve larger stores of amino acids in the re-pitched cultures (Miller *et al.*, 2013). The results from that study were aligned with results from Kobayashi and colleagues (2007), who found

a gradual increase in the concentration of FAN that remained in the beer as yeast was serial re-pitched eight times.

#### *Glycogen and Trehalose*

During the re-pitching process, yeast usually successfully adapts to the changing environmental factors. Glycogen and trehalose are two compounds that the yeast produces or utilizes, depending on the point in the re-pitching process, enabling them to adapt to this constantly changing environment (Section 1.1.4).

Glycogen levels in cells as they are repitched seem to be cell strain dependent and wort density dependent. A study investigated glycogen levels in the yeast cell prior to pitching and prior to re-pitching in two strains and in two different wort types, 10 °P and 15 °P (Kordialik-Bogacka and Diowks, 2013). Glycogen levels in freshly propagated yeast strain (308) were lower than glycogen levels in the re-pitched yeast. The second strain (B4) monitored in the two wort types did not show a statistical difference between freshly propagated and its successive re-pitched fermentation as the other strain did (Kordialik-Bogacka and Diowks, 2013). This demonstrated that the trends in glycogen levels in the cells for re-pitching was strain dependent. An earlier study monitored glycogen levels in cropped yeast and in propagated yeast, but analysed in the cells post fermentation (Jenkins *et al.*, 2003). In one strain monitored, there was a decrease in glycogen post fermentation in the earlier generations and in the later yeast generations, the glycogen levels were stable. Again this reinforced that trends with glycogen in re-pitching were strain dependent.

#### *Cell Age*

Studies have been completed investigating the age of the cells in the culture. The definition of ageing used here was the number of times that a cell had produced a daughter cell. This is commonly quantified by the number of bud scars on the cell (Hough, 1961). Ideally, in a healthy culture, the following ratio of cells would be expected: ½ virgin cells, ¼ mother with one budscars, ⅛ mother with two budscars, and so on (Steinkraus *et al.*, 2008). If cell growth is insufficient in the fermentations being serial re-pitched, the average cell age may become altered. In addition, work with multiple strains of *S. pastorianus* demonstrated that changes and stresses in the growth environment also influence the number of divisions and average age that yeast strains can achieve (Rodgers *et al.*, 1999; Powell *et al.*, 2000b; Maskell *et al.*, 2001, 2003).

One study on serial re-pitching demonstrated that the yeast cells did not progressively age (Bühligen *et al.*, 2014). This study was done with a strain of *S. pastorianus* in three different breweries and selecting a crop in the middle of the settled

yeast as would be done when warm cropping yeast for re-pitching (Bühligen *et al.*, 2014).

The section of yeast collected from the cone for serial re-pitching is important because the average cell age changes between different sections of the cone (Deans *et al.*, 1997; Lawrence *et al.*, 2013). In order to maintain consistent fermentations with a healthy culture of yeast, the section of the cone for yeast cropping needs to be selected accordingly. One study mentioned that there may be older cells in the bottom of the cone that form during cell flocculation (Barker and Smart, 1996). This is logical because older cells are more flocculent than younger cells (Powell *et al.*, 2003). A later study was published showing that older cells were actually found at a higher frequency in the middle of the cone and lower in the bottom (Powell *et al.*, 2004). In fact, the average age of cells, defined by the average number of bud scars on a cell in the culture, that were first removed were 1.17 or 1.21 depending on if warm cropped or cold cropped was used. The highest average number of bud scars was 1.6 or 1.47 and found in the middle section (Powell *et al.*, 2004). A recent study (Lawrence *et al.*, 2013) found the same results that agreed with the work of Deans and colleagues (1997) that older cells were found at the bottom of the crop and younger cells were found in the middle. Interestingly, this study also showed that older cells were found on the top of the crop (Lawrence *et al.*, 2013). This discrepancy remains to be elucidated, however alterations in flocculative ability, cell physiology and morphology as cells age may provide an explanation. Additionally, mixing in the cone may occur from heat generated from the flocculated yeast (Cahill *et al.*, 1999), and this could also contribute to the discrepancies observed. Finally, this could also be a strain specific response. A complete understanding of the distribution of cell ages in the cone is important for maintaining a healthy yeast culture with a healthy cell age.

### *Cell Health*

Viability and vitality is frequently measured to assess the yeast culture health. Vitality refers to a yeast cell's physiological capabilities and viability describes the percentage of a yeast culture that is alive (Kwolek-mirek and Zadrag-tecza, 2014). Cell health may indicate problematic fermentations. Dihydrorhodamine (DHR) fluorescence intensity was analysed which can estimate the Reactive Oxygen Species (ROS) in the cell. It measures ROS with a fluorescence dye that reacts with superoxide (Henderson and Chappell, 1993). It was analysed for each crop prior to fermentation. The intensity increased for the first four crops and then appeared to have stabilised at the same

intensity for the proceeding four crops. Similar results were found using OXN fluorescence (lipophilic and anionic) (Kobayashi *et al.*, 2007).

Jenkins and colleagues assessed the viability of different crops of lager yeast and compared it to propagated yeast (Jenkins *et al.*, 2003). It showed that for four different yeast strains, two of the strains showed a decreased viability and the other two remained stable as the yeast was re-pitched. The same study also showed that three of the four strains analysed showed impaired membrane integrity in the re-pitched yeast shown by 1-Anilino-8-naphthalene-sulfonic acid (MgANS) (Jenkins *et al.*, 2003).

### *Genetics*

Molecular biology techniques can be used to indicate whether two yeasts are genetically similar. This can be done by investigating the genotype or the phenotype of the yeasts. In serial re-pitching, these techniques have been used to determine whether the yeasts changed. Powell and Diacetis reported that a fresh culture of yeast and then the same culture of yeast, after re-pitching 135 times, exhibited identical phenotypes (Powell and Diacetis, 2007). This was with a 245 hL wort fermentation starting at 10 °P where the yeast from the fermentation was used for bottle conditioning. These authors also demonstrated that there was no variation in the DNA sequences investigated between an ale strain of yeast culture and the same yeast culture that had been re-pitched 98 times. This indicated no difference between fermentation characteristics with the ale type strain (Powell and Diacetis, 2007). Genetic drift can occur spontaneously, however this data suggests some strains appear to stay genetically conserved during serial re-pitching (Powell and Diacetis, 2007).

Similarly, another industrial study monitored mRNA expression in *S. pastorianus* during serial re-pitched fermentations, which had been subjected to twenty successive rounds of fermentations. This study found that the genetic expression was stable throughout the cycle (Bühlig *et al.*, 2013). Another study showed that there was a change in gene expression for upregulation of trehalose and glycogen after the 6<sup>th</sup> run (Bühlig *et al.*, 2014).

### *Petite Mutants*

Previously published literature suggests that petite mutations accumulate with serial re-pitching (Jenkins *et al.*, 2009). This study found petites at a rate of 12 % of the population, which was the highest frequency observed in the study, with the highest generation number. The highest rate of petites found in other strains with serial re-pitching were 5, 7, and 9 %. Additionally, the authors observed that the highest occurrence of petite mutations was located in the middle portion of the cone, the section



collected for re-pitching (Jenkins *et al.*, 2009). Another study investigated the accumulation of petite mutations with serial re-pitching and found no increase in relation to petite mutation frequencies with the generation number. The highest frequency observed in this study was 0.55 % (Lawrence *et al.*, 2012). The authors postulated that the differences observed between the two discussed studies were due to differences in cropping methods between the studies (Lawrence *et al.*, 2012).

Lawrence and colleagues (2013) looked directly at the yeast cells that had flocculated in the cone by observing the petite mutations frequency, cell viability, cell density and age at 10 hL intervals from the collected yeast. The younger cells were found in the middle of the crop and at the top. Whereas older cells were found at the bottom of the crop and just before the top. The distribution of petite mutant frequencies in the cone varied depending on the section. In a 70 hL sample crop, the largest percentage of petite mutations were found at 30 hL and 60 hL at concentrations of 1.22 and 1.56 % petites. The other harvested sections of yeast remained under 0.5 % petites (Lawrence *et al.*, 2013).

Other recent studies have been undertaken that investigated petite mutations and their frequency in the cone of the fermenter. These studies found that petite mutations accumulate in the center of the cone with a lager strain of yeast. The hypothesis behind this finding is that the temperature in the center was warmer, influencing petite mutation. Generally, even when cone is cooled, the temperature in the center of the cone will rest between 6 and 8 °C. When brewers collect yeast, they collect from the center of the cone which could cause the culturing of higher frequencies of petite mutations as serial repitching is extended (Jenkins *et al.*, 2009). Lawrence and colleagues (2013) observed that petite mutations in the cone were likely at the same location as the aged cells in the population. The authors speculated that the aged cells have less resistance to stresses that cause mtDNA damage therefore subjecting the cells to petite induction (Lawrence *et al.*, 2013). In the cone, aged cells are most likely found in the center (Powell *et al.*, 2004), therefore this study showed that general beliefs about aged cells flocculating first in the fermentation and settling at the bottom were misguided. As well, this study demonstrated that larger cells tend to settle first at the end of fermentation and smaller cells last. Additionally, Gibson and colleagues (2008c) demonstrated that the increase in petite levels that occurs as the yeast population ages during re-cropping, reduces the fermentation quality of the yeast.

#### **1.4.4 Yeast Storage Between Fermentation**

When yeast is to be stored, the collected cells are kept in a sample of the beer they had fermented, and held ideally around 2 °C (Boulton and Quain, 2007). During this time, the yeast is exposed to challenging conditions: limited nutrients, ethanol, and cold temperatures. The yeast is held in these conditions until pitched into the subsequent fermentation. As yeast storage times increase, glycogen and trehalose levels in the yeast cells decrease, as does the viability (Somani *et al.*, 2012). When glycogen levels in the yeast cell are low, the rates of uptake for Class B and C amino acids in fermentation may be negatively impacted (Pickerell *et al.*, 1991).

Somani and colleagues (2012) investigated the impact of different storage temperatures on yeast quality. These authors found there was no difference in yeast viability, glycogen and trehalose levels in the cell, and ratio of saturated to unsaturated fatty acids in the cell membrane between 4 and 10 °C yeast storage temperatures over 72 hours. The storage temperature did influence the yeast's viability when stored at 25 °C compared to 4 and 10 °C.

### **1.5 Aims and Objectives**

The aim of this thesis was to investigate factors that may impact the extent that a yeast culture may be re-pitched.

The first objective determined whether a yeast culture in industrial fermentations was re-pitched to its optimal number. The process must be tailored to each strain and fermentation in individual breweries. The hypothesis was that even though breweries discard their yeast culture after a pre-determined number, the fermentation profile and flavour profiles would not indicate the yeast culture should be discarded. Two case studies were completed: one ale strain and one lager strain (Chapter 3 and 4).

Multiple laboratory-scale studies were undertaken. One complication with serial re-pitching is the accumulation of mutated cells in the cropped culture (Jenkins *et al.*, 2009). Previously published literature demonstrates that high frequencies of mutated cells impact fermentation (Ernandes *et al.*, 1993). However, industrial brewing situations have been demonstrated to find natural occurrences of mutated cells in concentrations below 4 %, not considering serial re-pitching (Jenkins *et al.*, 2009; Lawrence *et al.*, 2013). The impact that less than 10 % petite mutations in the pitched yeast culture has on fermentation quality was investigated. It was hypothesized that if the occurrence of petites were to be below 10 % of the population, their presence would

not affect the density attenuation during fermentation or create aberrant flavour profiles (Chapter 6).

Depending on the demand to produce a specific brand of beer in a brewery, the number of days that yeast is stored between fermentations may change. Researchers have previously explored the impact of yeast storage times and temperatures on yeast viability and vitality (McCaig and Bendiak, 1985b; Rhymes and Smart, 2001; Somani *et al.*, 2012). However, literature on the impact of storage time on the physical fermentation is limited (Sall *et al.*, 1988). It is recommended that the best scenario for yeast storage is to use the culture within 1 to 3 days (White and Zainasheff, 2010). It was hypothesized that if the yeast storage time varied by 48 hours, the sugar consumption rates by the yeast during fermentation would not be affected but the levels of flavour compounds produced would change significantly (Chapter 7). Additionally, a further hypothesis was proposed that glycogen levels would be higher and trehalose levels would be lower in yeast cultures that were stored for a shorter time.

During serial re-pitching, consistent initial dissolved oxygen levels between fermentations are difficult to attain and thus may vary between serial re-pitched fermentations. The impact of various initial levels of dissolved oxygen in the wort on fermentation were investigated. Typical dissolved oxygen levels for 12 °P worts are between 6 – 8 mg/L for both ale and lager yeast (O'Rourke, 2002). It was hypothesized that low dissolved oxygen levels (3 mg/L) would exhibit a slower density attenuation during fermentation than optimal (8 mg/L) and with highly oxygenated wort (16 mg/L) (Chapter 8). Additionally, it was hypothesized that the varying levels of dissolved oxygen would have an impact on esters, higher alcohols, and vicinal diketone levels post fermentation. Last, if the initial dissolved oxygen level in the wort was less than 6 – 8 mg/L, the yeast cells would contain significantly less concentrations of unsaturated fatty acids and sterols post fermentation.

## Chapter 2 – Methodology

### 2.1 Yeast Strains and Long Term Storage

*Saccharomyces pastorianus* SMA (VLB Berlin, Germany) and *S. pastorianus* W34/70 (Weinstephan, Germany) were stored at 4 °C on YEPD agar slopes (10 % w/v yeast extract, 20 % w/v bacteriological peptone, 20 % w/v D-glucose, and 15 % w/v agar No 1. from Fisher Scientific, Loughborough, UK) in a universal test tube.

To prepare the slopes, the yeast stock was streaked onto YEPD agar plates from the existing YEPD agar slope. This step was completed as a precaution to check for contamination. The YEPD agar plates were incubated for 24 hours at 30 °C and observed for bacterial contamination. Common bacterial contamination found in beer are *Lactobacillus brevis*, *Pediococcus damnosus*, *Acetobacter* species, and some enterobacteriaceae (Vriesekoop *et al.*, 2013). If no bacterial contamination was present, the plates were incubated for 48 hours longer to let yeast colonies form. Three to four colonies were taken aseptically and streaked onto a prepared slope. This was done to minimize variation between fermentations. The slopes were prepared by pouring approximately 10 mL YEPD agar into a universal tube. The universals with the agar were sterilized. In this thesis, all media, glassware, or materials that were sterilized were autoclaved at 121 °C and 15 psi for 15 minutes unless otherwise specified. The sterilized agar was left to solidify on a slope in the universal. Once a slope was inoculated with yeast, it was incubated at 30 °C for three days. The universals were sealed with parafilm and stored at 4 °C until needed or re-sloped.

### 2.2 Wort Preparation

#### 2.2.1 Batch #1501

The wort was produced using the International Centre for Brewing and Distilling's (ICBD) 2 hL pilot scale brewhouse (Briggs of Burton, Burton on Trent, UK). To prepare the grist, 36.40 kg of extra pale ale malt (Crisp Malting Group, Fakenham, UK) was finely ground with an Essex Major hammer mill (Christy Hunt Ltd., Suffolk, UK). All malt was stored at 11 °C. Once milled, the grist was transferred into the mash tun with 102 L of 71.4 °C water at a liquor : grist ratio of 2.8 : 1. The initial temperature of the mash was 64.9 °C, which was held for 60 minutes. After this temperature rest, the temperature rose at 1 °C/minute to 75.0 °C. Once 75 °C was reached, the mash was transferred to a Meura mash filter (Meura, Tournai, Belgium). The run off from the mash filter took 44 minutes. The run off rate started at 390 L/h and ended at 225 L/h. Near the end of the mash filtering step, 44 L of water was sparged into the mash tun. In total 228 L of wort was collected before the boil. The wort was

boiled for one hour. At the start of the boil, 83.4 g of pelleted Columbus hops (John I Hass Inc., Washington DC, USA) were added to reach 20 IBU. The alpha acid content of the Columbus hops was 13.7 %. At the beginning of the boil, the density was 12.01 °P and concentrated to 13.39 °P at the end of the boil. Post boiling, the wort was cooled and transferred to 250 mL and 2 L polypropylene wide mouth bottles that were rinsed with 2 % peracetic acid. The bottles were stored at – 20 °C until needed.

### **2.2.2 Batch #1521**

This batch of wort was also produced on the ICBD 2 hL brewery kit. To prepare the grist, 25.50 kg of extra pale ale malt (Crisp Malting Group, Fakenham, UK) was finely ground with an Essex Major hammer mill (Christy Hunt Ltd., Suffolk, UK). The grist was transferred to the mash tun with 71 L of water at 46.8 °C and a liquor : grist ratio of 2.8 : 1. The first temperature rest was 44.9 °C, which was held for 30 minutes. The mash temperature rose at 1 °C/minute to 64.9 °C and the temperature was held for 60 minutes. Finally, the temperature was increased to 74.9 °C at 1 °C/minute before being transferred to the mash filter (Meura, Tournai, Belgium). After 8 minutes of mash filtering, 54.5 L of sparge water was added to the mash tun. After 24 minutes of mash filtering, an additional 14.5 L was added and after 28 minutes, 15.6 L was added. The temperature of the sparge water was 75.8 °C. At the beginning of mash filtering, the run off rate started at 380 L/h and ended after 38 minutes at 204 L/h. In total 130 L of wort was collected before the boil. The wort was boiled for 1 h. At the start of the boil period, 58.4 g of pelleted Columbus hops (John I Hass Inc., Washington DC, USA) with an alpha acid content of 13.7 % were added to reach 20 IBU. The initial density pre-boil was 12.43 °P and concentrated to 14.22 °P by the end. After boiling, the wort was cooled and then transferred into 250 mL and 2 L polypropylene wide mouth bottles previously rinsed with 2 % peracetic acid. The bottles were stored at -20 °C until needed.

### **2.2.3 Batch #1637**

The last batch of wort was also prepared on the ICBD 2 hL brewery kit. The grist was prepared by finely grinding 25.50 kg of extra pale ale malt (Crisp Malting Group, Fakenham, UK) with the Essex Major hammer mill (Christy Hunt Ltd., Suffolk, UK). The grist was transferred into the mash tun with 71 L of 48.8 °C water at a liquor : grist ratio of 2.8 : 1. During mashing, the first temperature rest was 45.4 °C for 30 minutes. The temperature increased at 1 °C/minute to 64.9 °C and was held for 60 minutes. Before transferring the mash to the mash filter, the temperature was increased at 1 °C/minute until it reached 74.3 °C. The run off rate for the mash filter using the

Meura mash filter (Meura, Tournai, Belgium), started at 400 L/h and ended after 38 minutes at 355 L/h. After 8 minutes of mash filtering, 52.9 L sparge water was added to the mash tun and at 20 minutes, 70.7 L was added. At the end of filtering at 38 minutes, 16.0 L of sparge water at 75.8 °C was added. In total 148.9 L of wort was collected. The wort was not boiled, but was immediately transferred into 2 L polypropylene wide mouth bottles. Extra steps were taken at this point to remove the trub before storage. The bottles were autoclaved (121 °C, 15 psi, 15 minute) allowing the hot break to form. After autoclaving, the bottles were stored at 4 °C for 16 h. and then moved to – 20 °C for 26 h. This process allowed the cold break to form. After storage at – 20 °C, the wort was held at 4 °C until the wort thawed. The thawed wort was centrifuged in 1 L centrifuge bottles at 5500 rpm for 5 minutes. The trub formed a pellet and the wort (supernatant) was transferred into the 2 L bottle. This step took three days to remove the trub from the entire batch of wort. The wort was held at 4 °C during this time. Once the trub was removed from the entire batch of wort, the bottles were stored at -20 °C until needed.

## **2.3 Miniature Fermentations**

### **2.3.1 *Yeast Propagation***

Yeast was propagated according to the yeast culture section in the methods of ASBC Yeast-14. To start the initial propagation, stored yeast was aseptically transferred with a flamed and cooled metal loop from the slope to a 125 mL Erlenmeyer flask containing 50 mL of sterile YEPD media (10 % w/v yeast extract, 20 % w/v bacteriological peptone, and 20 % w/v D-glucose all from Fisher Scientific, Loughborough, UK). The flask opening was fitted with a sterile foam bung to allow air to pass and block airborne bacteria from contaminating the culture. The inoculated media was incubated in a shaken incubator (100 rpm, 30 °C) for 24 hours. After 24 hours, the inoculated media was aseptically transferred to 50 mL centrifuge tubes and centrifuged at 3 000 x g (avg.) for three minutes in a fixed angle centrifuge.

Next, the cells were ‘washed’ three times as per ASBC Yeast-14. This step was completed between propagation steps and between re-pitched fermentations. The yeast was washed to remove potential residual sugars and ions on the exterior of the cell. This ensured that the conditions between fermentations remained consistent at the start of fermentation. Additionally, this ensured a prompt start to the fermentation; this was necessary with the small-scale fermentations because yeast settling before fermentation commenced could be problematic. To wash the cells once, the supernatant was discarded and approximately 40 mL of sterile distilled water was added to the yeast

pellet. The centrifuge tube was mixed using a vortex mixer to re-suspend the yeast pellet. Once achieved, the yeast slurry was centrifuged at 3 000 x (avg.) for three minutes to form the yeast pellet, after which the supernatant was discarded. This was repeated two more times.

The yeast slurry was counted by using a haemocytometer and halogen light microscope (Section 2.4) or by using an Aber Countstar (Aber Instruments, Aberystwyth, UK) (Section 2.5). Once the pitching volume was calculated, the cells were inoculated at  $1.5 \times 10^7$  cells/mL into two 250 mL Erlenmeyer flasks containing 100 mL of YEPD broth and fitted with a sterile foam bung. The culture was placed in a shaken incubator (100 rpm, 30 °C) for 24 hours. After 24 hours, the culture was removed from the incubator and the yeast was washed three times, as previously described. The yeast was then counted to determine the concentration of yeast in each yeast slurry and the viability was assessed using methylene blue (Section 2.4 or 2.5). This prepared a yeast culture for fermentation.

### **2.3.2 Fermentations**

Three days prior to pitching day, frozen wort (Section 2.2) was removed from the freezer and placed in a fridge at 4 °C. The day before the beginning of the fermentation, the wort was transferred into 2 L Erlenmeyer flasks. The flasks with the wort were covered with an aluminium foil cap, sterilized, and then stored in a fridge at 4 °C overnight. Before fermentation, the wort was transferred into 500 mL pre-sterilized centrifuge bottles (Beckman Coulter, High Wycombe, UK) and centrifuged with a Beckman Coulter centrifuge on a fixed angle rotor for 500 mL bottles spun at 3371 x g (avg.) for 20 minutes. The trub formed a pellet and the supernatant (wort) was carefully decanted from the bottles back into the Erlenmeyer flasks leaving the pellet in the bottles. The wort density was adjusted to 12.6 °P with sterile distilled water and the volume was adjusted to 90 % of the final volume. The other 10 % volume compensated for the volume of pitched yeast to be added and the remaining volume adjusted with distilled water. Once the density was achieved, 4 % w/v of D-glucose was added to achieve 16.1 °P. The volume utilized for this calculation was the final volume. Five minutes before pitching, the wort was oxygenated with sterile oxygen for five minutes.

The yeast culture was added at  $1.5 \times 10^7$  cells/mL., and sterile distilled water was added until the appropriate final volume was reached. Next, 20 mL of wort was transferred into test tubes (18 mm x 20 mm) with a sterile boiling chip at the bottom and a sterile foam bung was placed in the opening. The tubes were placed in a water bath at 21 °C to ferment. At hours 0, 1, 6, 22, 26, 30, 46, 50, 54, 70, 74, and 78, three small test

tubes were removed for destructive sampling. For sampling, a transfer pipette took a sample of the beer from approximately 1.5 cm from the top. The transferred sample was then placed into a disposable 2 x optical acrylic cuvette (Sarstedt, Nümbrecht, Germany). The absorbance was measured at 600nm. The sample that remained in the test tube was filtered through Whatman #4 filter. A 20 mL syringe was used to uptake 8 mL sample, and that was used as a rinse through the Anton Paar DMA 4500 with alcoalyzer beer ME (Anton Paar Ltd., St. Albans, UK). After the rinse, 10 mL of sample was inserted into the machine. The density (°P), and alcohol (ABV) measurements were recorded. The sampling was completed in triplicate for each time point.

### 2.3.3 *Density and Yeast in Suspension Analysis*

The absorbance was fitted to a tilted Gaussian fit (Equation 2.1),

$$Abs_{600} = R \cdot t + A \cdot e^{-\frac{1}{2}\left(\frac{t-\mu}{\sigma}\right)^2}$$

(Equation 2.1)

where  $Abs_{600}$  is the absorbance at any time  $t$ ,  $R$  is the slope,  $\mu$  is the midpoint,  $\sigma$  is the width factor, and  $A$  is the absolute amplitude. This statistical analysis was performed using Graphpad Prism 2016 (GraphPad Software, La Jolla, USA). An F-Test was used to determine if two yeast in suspension curves were significantly different. For all statistical analysis performed in this thesis, tests with  $p > 0.05$  were considered significant unless otherwise stated.

The density attenuation was monitored with a four-parameter nonlinear regression from the methods in ASBC Yeast-14 (Equation 2.2),

$$P_t = P_e + \frac{P_i - P_e}{1 + e^{-B(t-M)}}$$

(Equation 2.2)

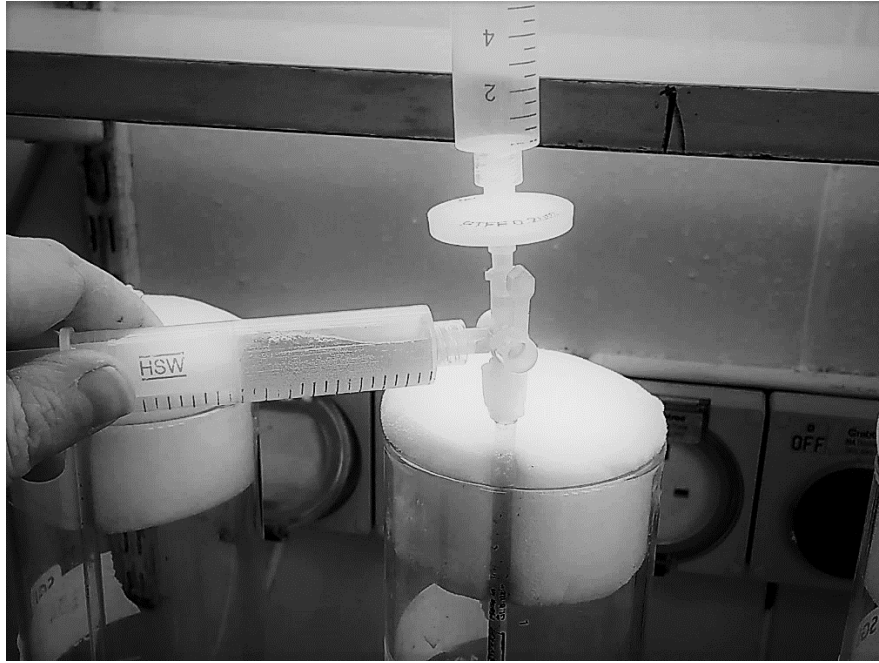
where  $P_t$  is the extract value at time  $t$  (°P),  $P_e$  is the final asymptotic extract value (°P),  $P_i$  is the initial asymptotic extract value (°P),  $B$  is a function of the slope at the time of inflection (°P/h) and  $M$  is the time it takes to reach the inflection point (h). An F-Test was used to determine whether two density attenuation curves were significant. Yeast in suspension and density attenuation modelling was performed using Graphpad Prism.



## 2.4 Graduated Cylinder Fermentations

Prepared wort (Section 2.2) was thawed for three days at 4 °C in 2 L bottles. Once thawed, the wort was transferred to 2 or 4 L Erlenmeyer flasks, autoclaved (121 °C, 15 minutes, 15 psi), and stored at 4 °C overnight. The wort density diluted using sterile distilled water until it reached 12.6 °P. Once diluted, the volume was adjusted to 90 % of the final volume. The other 10 % volume compensated for the mL of pitched yeast to be added and the remaining volume adjusted with distilled water. The wort was oxygenated for five minutes by bubbling pure oxygen at approximately 3 psi through the wort.

The yeast was pitched directly into the 2 or 4 L Erlenmeyer flasks and stirred briefly with a sterile magnetic stirring rod. Once evenly mixed, 1 L of the pitched wort was transferred into a 1 L graduated cylinder and fitted with a foam bung. A sample port was placed into the side of the foam bung (See Figure 2.1). The sterile sample port was a 3-way stop cock with a 1 mL glass pipette attached to one end with silicon tubing. The 1 mL glass pipette was inserted into the fermenting beer. The other two sample ports were open to the outside of the fermenter. To take a sample, two syringes were connected to the three-way stopcock sample port (Figure 2.1) and one of the two syringes had a 0.2 µm filter attached to it. The three-way stopcock was opened so that one syringe could take a 15 mL sample. Once collected, the three-way stopcock was turned so that it was open to the syringe with the attached sterile filter. Sterile air was plunged into the fermenter so that no fermenting beer remained in the interior of the 1 mL pipette. Once this was achieved, the stopcock was closed to the fermenter. Once closed, the stopcock was sprayed with 70 % ethanol. After one minute, sterile air was used to clear the ethanol from the inside of the stopcock. In between samples, the sample port was covered with aluminium foil so airborne contaminants would not settle inside the stopcock.



**Figure 2.1.** Sample port that allowed sampling without removing the foam bung.

A 15 mL sample of beer was removed at hour 0, 6, 24, 30, 48, 54, 72, 78, 96, and 102 during the fermentation. These times were chosen for adequate sampling to model the density attenuation and yeast in suspension trends, however sampling was limited to ensure the fermentation height did not drastically change. The height of the fermenter and medium volume is influential on the shear rate within the fermenter from CO<sub>2</sub> evolution (Lake *et al.*, 2008) The power of agitation on the fermentation by CO<sub>2</sub> bubbles is impacted by a number of parameters (Equation 2.3) (Delente *et al.*, 1968).

$$P = BQ_{CO_2}\rho gH_a \left\{ \left[ \frac{H_a + H_f}{H_f} \right] \ln \left[ \frac{H_a + H_f}{H_a} \right] - 1 \right\}$$

(Equation 2.3)

For this equation, B is the medium volume, Q is the rate of CO<sub>2</sub> evolution,  $\rho$  is the wort density, g is acceleration due to gravity, H<sub>a</sub> is atmospheric pressure, and H<sub>f</sub> is the fermenting height (Delente *et al.*, 1968). In these fermentations, the two parameters that changed were medium volume and fermenting height. Care was taken during sampling to consistently remove the same volume from the medium volume. With consistent sample times between fermentations, the changing height and changing medium volume was identical between all fermentations and was therefore not considered a confounding factor.

With the removed sample, a 3 mL aliquot was transferred into a disposable 2 x optical acrylic cuvette (Sarstedt, Nümbrecht, Germany). The side of the cuvette was tapped to ensure no bubbles were stuck on the sides and then the cuvette was placed into a Genesys 6 spectrophotometer (Fisher Scientific, Loughborough, UK). The absorbance was measured at 600 nm and recorded. Once completed, the sample in the cuvette and the remaining sample in the syringe were recombined and filtered through a Whatman #4 filter paper. This step removed yeast and degassed the sample. A handheld densitometer DMA 35 (Anton Paar Ltd., St. Albans, UK) was cleaned with distilled water and dispensed to empty the densitometer. A sample of degassed and yeast-free beer was taken up into the densitometer. The sample was held in the instrument until a measurement was stable. The sample times for these fermentations were hour 0, 1, 6, 24, 30, 48, 54, 72, 78, 96, and 102.

These density attenuation and yeast in suspension trends were modelled by using the Yeast-14 methods (Section 2.3.3). The cropping methods and storage procedures are described in individual chapters.

## **2.5 Cell Counting and Viability**

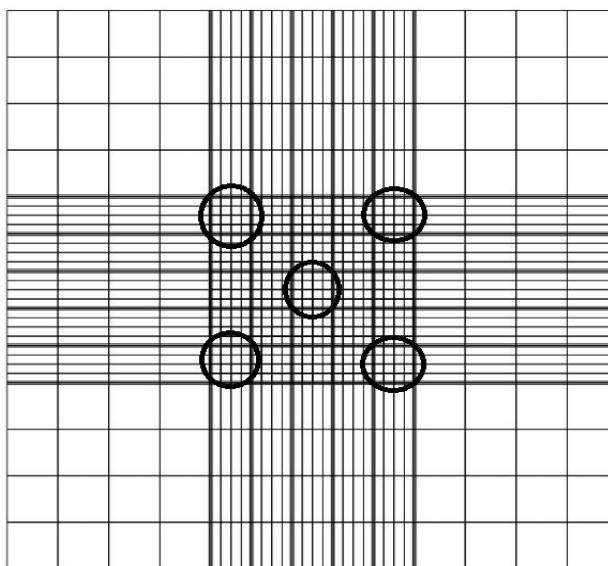
### ***2.5.1 Using a Haemocytometer***

To prepare and quantify yeast cell concentration with a haemocytometer, a modified method of ASBC Yeast-4 was followed. Yeast slurries were diluted to a concentration between  $1 \times 10^5$  and  $6 \times 10^7$  cells/mL with 0.5 M ethylenediaminetetraacetic acid (EDTA) in distilled water. Methylene blue solution (0.01 % w/v methylene blue and 0.2 % w/v sodium citrate tribasic dehydrate (Fisher Scientific, Loughborough, UK) were added to the slurry at a 1 : 1 ratio.

To prepare the haemocytometer, the coverslip was placed over the counting chamber and rested on the glass side supports. Using a fine-tip pipette with 20  $\mu$ L of the prepared yeast slurry was used to fill the chamber. The prepared slide rested for two minutes to let the yeast settle. Five of the 25 counting squares in the 1 mm<sup>2</sup> area were counted and multiplied by five to determine the number of cells in the entire 1 mm<sup>2</sup> area. The five squares counted were the four corners and the middle square (See Figure 2.2). The white and blue cells were scored to express % viability and cell concentration was also calculated (Equation 2.4).

$$\frac{\text{cells}}{\text{mL}} = \text{Total cells in 5 square ruled area} \times \text{dilution factor} \times (1 \times 10^4) \times 5$$

(Equation 2.4)



**Figure 2.2.** Counting chamber on the haemocytometer. The five circles identify the five squares that were counted for cell concentration determination.

### 2.5.2 Using the Aber Countstar

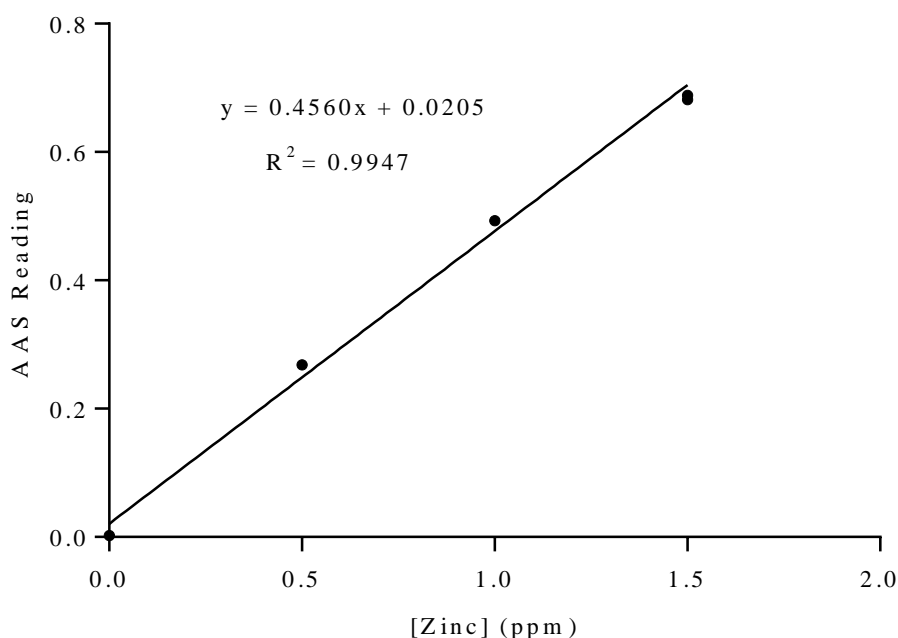
Yeast slurries were diluted to a concentration between  $1 \times 10^5$  and  $6 \times 10^7$  cells/mL with 0.5M ethylenediaminetetraacetic acid (EDTA) in distilled water. Methylene blue solution as described in Section 2.4 was added to the slurry at a 1 : 1 ratio. A 20  $\mu\text{L}$  aliquot of the methylene blue and yeast cell solution was pipetted into the well of the disposable Countstar slides. The slides were slightly tapped to ensure no air pockets were held within the well. After the slide rested for two minutes to let the yeast settle, the slide was placed into the Aber Countstar. The cell concentration, % viability, circularity index, width ( $\mu\text{m}$ ), and length ( $\mu\text{m}$ ) of the yeast culture was quantified using a computer software program (Aber Countstar, Aberystwyth, UK).

## 2.6 Zinc Determination in Wort

Zinc levels were determined by Perkin Elmer AAnalyst 200 with an air/nitrous oxide flame and a multi-element (Cu, Fe, Mn and Zn) hollow cathode lamp (PerkinElmer Inc., Waltham, USA). All glassware was acid washed, by using 2 %  $\text{HNO}_3$  (Fisher Scientific, Loughborough, UK) for at least 24 h. The glassware was rinsed with deionized water three times and dried in an oven prior to use.

All wort samples were centrifuged to ensure no solids remained in the wort. Each sample was acidified by adding 200  $\mu\text{L}$  69 % (w/v)  $\text{HNO}_3$ . Zn standard (Sigma

Aldrich Co. Ltd., Irvine, UK) was used to create a standard curve between 0 and 1.5 ppm in triplicate by diluting with deionized and distilled water (Table 2.2). A blank was also prepared with the same deionized and distilled water that was utilized to prepare the standard curve.



**Figure 2.3.** Zinc calibration curve with each concentration completed in triplicate.

The blank and standards were analysed and then the wort samples were analysed. The concentrations of Zn in the samples were quantified directly by the calibration curve correcting for the dilution factor. Each sample was completed in triplicate.

## 2.7 Free Amino Nitrogen (FAN) Analysis

To determine free amino nitrogen (FAN) levels in the wort, the ASBC Wort-12 method was followed. This method measured amino acids and ammonia, as well as some end-group  $\alpha$ -amino nitrogen in peptides and proteins in beer and wort (Gibson *et al.*, 2009).

Samples of wort were diluted 1 : 99 with distilled water and 2 mL was transferred to a labelled test tube. To prepare the calibration standard, 107.2 mg of glycine (Fisher Scientific, Loughborough, UK) was dissolved in 100 mL of distilled water by using a volumetric flask. Prior to running the method with the wort samples, the glycine solution was diluted 1 : 99 with distilled water. This standard contained 2 mg amino nitrogen/L. A 2 mL aliquot of the standard was transferred to an

appropriately labelled test tube. In addition to the standard and samples, a blank was performed that was 2 mL of distilled water in a labelled test tube. A 1 mL aliquot of ninhydrin colour reagent was pipetted into each test tube. The reagent consisted of 10 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.5 g ninhydrin, and 0.3 g fructose (all from Fisher Scientific, Loughborough, UK) in 100 mL distilled water in a volumetric flask. The pH of this reagent was between 6.6 – 6.8 and was stored at 4 °C in an amber bottle. After the ninhydrin reagent was added, the samples were heated in a boiling water bath for 16 minutes. Proceeding the incubation, the samples were transferred to a water bath at 20 °C for 20 minutes. After 20 minutes, 5 mL of a dilution solution was added to each sample. The dilution solution contained 2 g of dissolved  $\text{KIO}_3$  in 600 mL distilled water and 400 mL of 96 % ethanol (Fisher Scientific, Loughborough, UK). The samples were inverted five times and measured with a Genesys 6 spectrophotometer (Fisher Scientific, Loughborough, UK) at 570 nm against the prepared blank. The concentration of FAN was calculated from the absorbance measurements (Equation 2.5). The absorbance was measured within 30 minutes of adding the dilution solution. Each sample was prepared in triplicate.

$$FAN \left( \frac{\text{mg}}{\text{L}} \right) = \frac{\text{net absorbance of test solution}}{\text{net absorbance of glycine standard}} \times 2 \times \text{dilution}$$

(Equation 2.5)

## 2.8 Bud Scar Quantification with Calcofluor White Stain

Cells were stained with calcofluor and observed by fluorescence microscopy to determine the number of bud scars on each cell (Pringle, 1991). A 1 mg/mL stock solution of Calcofluor White Stain (Sigma Aldrich Co. Ltd., Irvine, UK) in distilled water was prepared. This solution was stirred for 5 hours at room temperature to ensure the dye was completely dissolved. The bottle for this solution was covered with aluminium foil to prevent light from reacting with the dye. The stock solution was stored in the dark at 4 °C.

Yeast cells were washed three times and re-suspended in distilled water at a concentration of approximately  $10^7$  cells/mL. A 1 mL aliquot of calcofluor stock solution was added to 2 mL of washed yeast. This solution was incubated in the dark for five minutes. Following the incubation time, the cells were washed three times by centrifugation with distilled water and then mounted on a microscope slide. The cells were observed under a Zeiss Axiophot fluorescence microscope (Cark Zeiss Ltd.,

Cambridge, UK) with a DAPI-FITC filter. The number of bud scars on a minimum of 100 cells was quantified and the average was reported.

## **2.9 Ester, Higher Alcohol, and Vicinal Diketone Quantification in Beer**

All flavour compound analyses were completed by the analytical laboratory in the School of Life Sciences, Heriot-Watt University. Esters, higher alcohols, and vicinal diketones (VDKs) were analysed using headspace gas chromatography (GC).

### **2.9.1 Gas Chromatography Conditions**

The column temperature was held at 43 °C for 2 minutes, increased to 86 °C at 1.5 °C/minute and then increased to 180 °C for 5.22 minutes with a head pressure of 25 psi. The temperature of the injection was held at 180 °C. The carrier gas was nitrogen at 100 mL/minute. to the Electron Capture Detector (ECD) and 32 mL/minute to the Flame Ionisation Detector (FID). The pressure for the carrier gas was held at 78 psi. A 200 µL sample was injected by using a Perkin Elmer HS40XL headspace auto sampler into a split injector packed with glass wool. The temperature was 180 °C and the split ratio was 56 : 1 with a split vent of 80 mL/minutes and septum purge of 3.3 mL/minute.

### **2.9.2 Esters, Higher Alcohol and VDKs**

Samples of beer were placed in a vial with 50 µL volume of internal standard that contained 200 mg/L 3-heptanone and 18.1 mg/L hexanedione in absolute alcohol and then the vial was sealed. The beer samples were incubated at 60 °C for 90 minutes. Samples were injected into the GC. The samples ran through a Chromepack CP-Wax-57-CB (dimensions 60m long, 0.25 mm i.d, 0.40 µm thick) column was used with a Hewlett Packard 589 - series II GC with split injector. A Flame Ionisation Detector (FID) and an Electron Capture Detector (ECD) were used depending on the flavour compound analysed. The VDKs were measured with the ECD and esters, higher alcohols and other flavour compounds were analysed with the FID. The temperature settings for the oven, needle, and transfer tubing were 60, 70, and 110 °C respectively. The column flow rate was set to 1.5 mL/minute. The concentrations were calculated either manually with the calibration curves and area peaks or with a computer based program.

## **2.10 Simple Carbohydrate Quantification in Wort and Beer**

All carbohydrate analyses were completed by the analytical laboratory in the School of Life Sciences, Heriot-Watt University. Glucose, fructose, sucrose, maltose and maltotriose concentrations were quantified, using High Performance Anion Exchange (HPAE). When the pH is high, sugars are ionised. This ionisation allowed

separation of simple carbohydrates by anion exchange. The detector that was used for sugar quantification was a pulsed amperometric detector.

Two columns were used: a 4 x 50 mm Dionex Carbopac PA-100 Guard column and a 4 x 250 mm Dionex Carbopac PA-100 column and a Dionex Pulsed Electrochemical Detector with a gold electrode (all from Dionex, Sunnyvale, USA). The instrumentation also included a Hewlett Packard 1050 autoinjector, and a Dionex eluent degas module. Depending on the expected carbohydrate concentration, samples were used as is or diluted to a 1 : 200 dilution. A 900 µL aliquot of sample was added to 180 µL of internal sample, mixed, and transferred to a 1.5 mL glass vial and capped. Prior to running the samples, a vial of fresh calibration standard with internal standard was run first and compared to the calibration at the end of each run. To run a sample, 20 µL of sample was taken up at 200 µL/minute. The flow rate was set to 1000 µL/minute and a pressure of 0.72 psi was maintained.

The chemicals used for the calibration standard were AnalR glucose, fructose, and sucrose (> 99 % pure), D(+)-Maltose Monohydrate and D(+)-Cellobiose (> 99 % pure), Maltotriose (all from VWR, Lutterworth, UK) and HPLC grade deionised water (< 18 Megohms/cm resistance) (Milli-Q-system).

## **2.11 Petites Determination by Triphenyl Tetrazolium Chloride (TTC)**

Cultures of yeast suspended in beer were counted (Section 2.5.2) to determine the concentration of viable yeast. A sample of yeast was serially diluted until a solution with approximately 750 cells/mL was achieved. This was repeated 10 times. A 0.1 mL aliquot of the diluted yeast was spread onto 10 YEPD agar plates (10 % w/v Yeast Extract, 20 % w/v Bacteriological Peptone, 20 % w/v D-Glucose, and 15 % w/v Agar No 1. from Fisher Scientific, Loughborough, UK) for each replicate. This step ensured that 10 replicates with a minimum of 500 cells each were enumerated for the TTC overlay method (Ogur *et al.*, 1957).

The plates were incubated at 30 °C. After three days, a solution of 20 mL of autoclaved (121 °C, 15 psi, 15 minutes) phosphate buffered agar (1.53 g/L potassium dihydrogen phosphate, 1.94 g/L disodium hydrogen phosphate, and 15 g/L Agar No. 1) containing 5 g/L TTC (all from Sigma Aldrich Co. Ltd., Irvine, UK) at 55 °C was slowly poured over the colonies. A 10g/L TTC solution in distilled water was separately prepared and autoclaved, because TTC becomes reduced when autoclaved in agar (Ogur *et al.*, 1957). The TTC solution was added to the phosphate buffered agar in equal



quantities at 55 °C prior to overlaying. The plates were incubated at 21 °C for 4 hours. The resulting cells with active mitochondria reduced the TTC to formazan that produced a red/pink colour. The cells with impaired mitochondria, i.e. petites, remained white. After 4 hours, the white and red/pink colonies were scored and reported as percentages.

### **2.12 Petites Determination by YEPGly and YEPD Agar Plates**

Cultures of yeast suspended in beer were counted (Section 2.5.2) to determine the concentration of yeast. A sample of yeast was serially diluted until a solution with approximately 750 cells/mL was achieved. This step was repeated three times. A 0.1 mL aliquot of the diluted yeast was spread onto autoclaved (121 °C, 15 minutes, 15 psi) YEPD agar plates (10 % w/v yeast extract, 20 % w/v bacteriological peptone, 20 % w/v D-glucose, and 15 % w/v Agar No 1. from Fisher Scientific, Loughborough, UK) and YEPGly agar plates (10 % w/v yeast extract, 20 % w/v bacteriological peptone, 20 % v/v glycerol, and 15 % w/v Agar No 1. from Fisher Scientific, Loughborough, UK) in triplicate. The cells were incubated for 3 days at 30 °C. Petite cells were unable to grow on nonfermentable substrates, i.e. glycerol. Therefore, the cells on the YEPGly plates were solely respiratory sufficient, while the cells on the YEPD plates were both respiratory sufficient and respiratory deficient. The % petites were calculated and the results were recorded.

### **2.13 Glycogen and Trehalose Analysis in Yeast**

Glycogen and trehalose were analysed by enzymatic digestion. The enzyme  $\alpha$ -amyloglucosidase degraded glycogen to glucose and the enzyme trehalase degraded trehalose to glucose. Once the compounds were anabolized, the glucose concentration was measured by glucose standard kits. The enzymes and glucose kits were from Sigma Aldrich Co. Ltd., Irvine, UK.

Yeast samples were washed three times (described in Section 2.3.1) with distilled and deionised sterile water. The cell slurries were diluted to approximately  $1 \times 10^9$  cells/mL. The cell concentrations were calculated (Section 2.5.2) and recorded. A 1 mL sample of yeast slurry of known concentration was transferred into a labelled 2 mL Eppendorf Safe-lock micro centrifuge tube. The samples were stored at – 20 °C until all samples were collected.

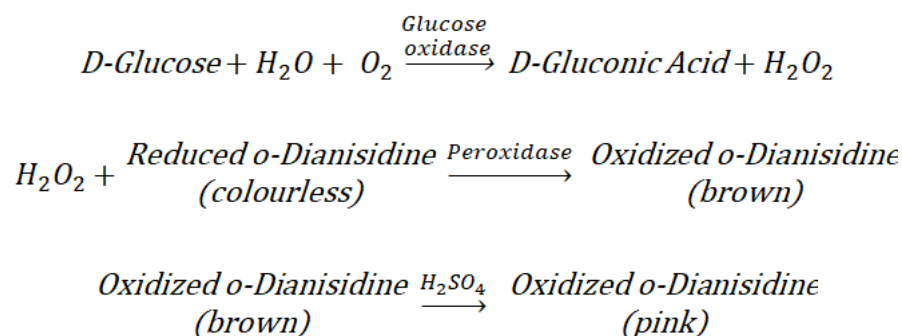
A modified method from Jenkins *et al.* (2003) was utilized. Samples were thawed for 3 minutes in a 4 °C water bath. A 125  $\mu$ L aliquot of 0.25 M sodium carbonate (Fisher Scientific, Loughborough, UK) in deionised water and 250  $\mu$ L of

deionised water were added to a 250  $\mu\text{L}$  aliquot of yeast in a new 2 mL Eppendorf micro centrifuge tube. The micro centrifuge tube was sealed and incubated at 95  $^{\circ}\text{C}$  for 2 hours to lyse the cells, inactivate cellular enzymes, and extract glycogen and trehalose (Schulze *et al.*, 1995). Samples were placed on ice and 300  $\mu\text{L}$  of 0.2 M sodium acetate (Fisher Scientific, Loughborough, UK) in deionised water and 75  $\mu\text{L}$  of 1 M acetic acid (Fisher Scientific, Loughborough, UK) in deionised water were added to each sample. This adjustment ensured that pH was optimal for enzyme function to degrade the glycogen or trehalose. The samples were shaken with a vortex mixer. A 500  $\mu\text{L}$  aliquot of sample was transferred to a new labelled 2 mL Eppendorf Safe-lock micro centrifuge tube leaving 500  $\mu\text{L}$  in the original micro centrifuge tube. A 10  $\mu\text{L}$  aliquot of trehalase (Sigma Aldrich Co. Ltd., Irvine, UK), isolated from porcine kidney, was transferred into one tube and 5  $\mu\text{L}$  of  $\alpha$ -amylglucosidase (Sigma Aldrich Co. Ltd., Irvine, UK), isolated from *Aspergillus niger*, was transferred into the other. These volumes ensured each sample contained 0.5 U/mL trehalase or 1.2 U/mL  $\alpha$ -amylglucosidase enzyme digestion (Parrou and François, 1997). The enzyme  $\alpha$ -amylglucosidase cleaves glycogen and in small quantities trehalose. However, with incubation above 55  $^{\circ}\text{C}$ , trehalose was not degraded and glycogen was degraded (Parrou and François, 1997). For this reason, the tube with  $\alpha$ -amylglucosidase to break down glycogen was incubated in a hybridization oven at 57  $^{\circ}\text{C}$ . The tube containing trehalase was placed in a shaken incubator at 37  $^{\circ}\text{C}$  to break down trehalose. The incubation time for both samples was 16 hours.

After the incubation period, the glucose was quantified by using glucose standard kits (Sigma Aldrich Co. Ltd., Irvine, UK). The samples in the micro centrifuge tubes were centrifuged at 4 000 x g for 3 minutes. For the glycogen samples, a 2.5  $\mu\text{L}$  sample of the supernatant was transferred into a clean test 10 mL test tube with 497.5  $\mu\text{L}$  of deionised water. A 25  $\mu\text{L}$  aliquot of supernatant from the trehalose samples was transferred into 475  $\mu\text{L}$  of deionised water. Additionally, to prepare a calibration curve, D-glucose (1.0 mg/mL in 0.1 % benzoic acid) was diluted with deionized water to prepare 0, 20, 40, 60, and 80  $\mu\text{g}$  glucose/mL solutions.

When all samples were diluted, an 'Assay Reagent' was prepared. To prepare the assay reagent, a capsule that contained glucose oxidase/peroxidase reagent (Sigma Aldrich Co. Ltd., Irvine, UK) was dissolved in 39.2 mL of deionized water. A 0.8 mL aliquot of 5 mg/mL of o-Dianisidine reagent (Sigma Aldrich Co. Ltd., Irvine, UK) was mixed with the glucose oxidase/peroxidase solution. This solution was the assay

reagent. A 1 mL aliquot of the assay reagent was added to each sample as well as a blank that contained deionized water. The reagent was added to each test tube at 20 second intervals. This precaution was done to ensure the incubation time was precisely 30 minutes for each sample. Once the reagent was added to all the samples, the tubes were incubated in a water bath at 37 °C. During the incubation period two steps of a three-step reaction took place. First, glucose reacted with water to form hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Secondly, H<sub>2</sub>O<sub>2</sub> reacted with o-Dianisidine to produce oxidized o-Dianisidine (Equation 2.6). After a 30 minute incubation, 1.0 mL of 12 N H<sub>2</sub>SO<sub>4</sub> was added to each sample at 20 second intervals, which initiated the third step in the reaction. Hydrogen peroxide created a stable form of oxidized o-Dianisidine that was pink (Equation 2.6). This stopped the reaction. The samples were mixed thoroughly then transferred into a cuvette. The absorbance was measured on a Genesys 6 spectrophotometer (Fisher Scientific, Loughborough, UK) at 540 nm against the blank and recorded. The concentration of glucose was quantified using a calibration curve.



(Equation 2.6)

## 2.14 Yeast Lipid Analysis

### 2.14.1 Sample Preparation Prior to Lipid Isolation

Samples for lipid analysis were taken at two time periods during fermentation. One sample was taken at time zero and another sample was taken at hour 102 from the cropped yeast. At time zero, a 90 mL sample of beer was removed from the fermenter immediately after the yeast was inoculated into the wort at  $1.5 \times 10^7$  cells/mL. The yeast was centrifuged (3 000 x g (avg.), 3 minutes) in two 50 mL sterile centrifuge tubes. The supernatant was discarded and the yeast in both tubes were re-suspended in approximately 15 mL of deionised and distilled water. The two slurries of yeast were pooled into one tube and the empty centrifuge tube was rinsed twice to ensure all yeast was transferred into the one centrifuge tube. The cells were then washed three times (Section 2.3.1). After the final washing step, the supernatant was discarded and the

yeast pellet was re-suspended in 1 mL of distilled and deionised water. The slurry was transferred into a 10 mL wide-mouthed glass vial. The centrifuge tube was rinsed two more times with 1 mL of distilled and deionised water and transferred to the 10 mL glass vial. The glass vial was weighed with an analytical balance before and after the sample was added. A plastic lid was placed on the glass vial and the collected sample was frozen at  $-60^{\circ}\text{C}$  until all samples were collected.

All samples were placed in a freeze dryer (Edwards Super Modulyo, Fisher Scientific, Loughborough, UK) to dehydrate the samples. The samples were stored in the freeze dryer for one week to ensure all moisture was removed. Immediately after the samples were removed from the freeze dryer, the samples were weighed with an analytical balance.

### ***2.15.2 Isolation of Lipids from Yeast***

All glassware and lids were sonicated for 10 minutes in water with detergent. The glassware and lids were rinsed with water to remove residual soap, followed by an acetone rinse and dried overnight in an oven. Finally, the glassware and lids were rinsed with dichloromethane (Fisher Scientific, Ottawa, Canada) and left in a fume hood for approximately 15 minutes until the dichloromethane evaporated.

The internal standards 5 $\alpha$ -cholestan-3 $\beta$ -ol (Sigma Aldrich, Darmstadt, Germany) and 23:0 (Sigma Aldrich, Darmstadt, Germany) were pipetted into a 10 mL round bottom test tube. The solvent was evaporated with a 'N-EVAP' nitrogen evaporator (Organomation Associates Inc., USA) at approximately 4 litres per minute of nitrogen in a water bath at  $32^{\circ}\text{C}$ .

The vial that contained dried yeast (Section 2.15.1) was weighed on an analytical balance to determine the weight from the atmospheric moisture that was absorbed by the sample. A 50-100 mg sample of dried yeast was weighed and added into the test tube with the internal standards using an analytical balance. A 2 mL aliquot of 95 % v/v ethanol (Fisher Scientific, Ottawa, Canada) in distilled water and 2 mL of 50 % w/v potassium hydroxide (Fisher Scientific, Ottawa, Canada) in distilled water were added to the test tube. A Teflon lined cap was placed on the tube so that a seal was formed and mixed by a vortex mixer. The sealed samples were placed on a heating block at  $100^{\circ}\text{C}$  for one hour. This step lysed the cells and formed fatty acid salts. After incubation, the test tubes were placed in the freezer for five minutes to cool. A 4 mL aliquot of hexane was transferred with a clean 10 mL glass pipette and transfer bulb into the test tube. The test tube was mixed thoroughly with a vortex mixer and the test tube

was centrifuged (240 x g) with an IEC HN SII centrifuge (GMI Inc., Ramsey, USA) for approximately two minutes. This allowed for separation of the aqueous and organic layers. The organic layer was recovered by using a transfer pipette and bulb, and was dispensed into a new 10 mL test tube. The organic layer contained the yeast sterols. Another 4 mL aliquot of hexane was dispensed into the original test tube, mixed by vortex mixer, and centrifuged (240 x g) for approximately two minutes. After separation of the aqueous and organic layer, the organic layer was recovered. It was combined with the previously recovered organic layer into the other test tube. The organic/aqueous interface in the original test tube was removed to ensure that only the aqueous layer remained. This test tube was saved for a later step because the test tube contained the fatty acid salts.

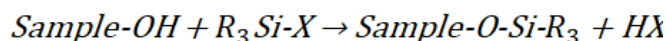
Approximately half of the volume of hexane from the test tube that contained the yeast sterols was evaporated. The hexane was evaporated by streaming nitrogen through the test tube while heated in a water bath. When approximately half of the hexane was evaporated, 3 mL of distilled water was transferred into the test tube by using a clean 10 mL glass pipette. The test tube was mixed with a vortex mixer and centrifuged at 160 x g for 10 minutes. The hexane layer was transferred into a newly labelled 10 mL test tube that contained approximately 5 g of sodium sulphate anhydrous. This step ensured that insoluble or partially soluble compounds in hexane, were removed from the organic layer. The test tube that contained the organic layer and salt was mixed in the test tube and the solvent was transferred into a new clean 10 mL test tube that was free of all sodium sulphate crystals. This test tube contained the isolated sterols.

The aqueous layer in test tube #1 was transferred to a 40 mL test tube taking care not to collect the yeast pellet. A 4 mL aliquot of deionised water was transferred into test tube #1 and the tube was mixed with a vortex mixer. The tube was centrifuged at  $\frac{3}{4}$  speed for approximately 5 minutes and the aqueous layer was transferred into the 40 mL test tube. A final 4 mL aliquot of deionised water was added and recovered into the 40 mL test tube. A single drop of phenolphthalein (0.01g/mL) was added to the 40 mL test tube that turned the aqueous layer pink meaning the solution was above pH 9 (Lo *et al.*, 2015). Sulfuric acid (6M) was added drop-wise until the solution turned a clear/yellow colour. This colour change happened when the pH was below 8. An extra drop of sulfuric acid was added to ensure the pH was below 7. At this pH, the fatty acid salts broke up and the free fatty acids became soluble in the organic layer. A 4 mL aliquot of hexane was added to the 40 mL tube, and the tube was mixed using a vortex

mixer. The test tube was centrifuged at 240 x g for approximately two minutes. A transfer pipette was used to transfer the hexane layer into a new 10 mL test tube that contained approximately 5 g of sodium sulphate anhydrous. Another 4 mL of hexane was added to the 40 mL test tube. The tube contents were mixed using a vortex mixer and were centrifuged at 240 x g for approximately two minutes. The hexane layer was transferred into the 10 mL test tube that contained the sodium sulphate. Finally, the hexane layer in the tube with the sodium sulphate was transferred into a new 10 mL test tube free from sodium sulphate crystals. This test tube contained the isolated fatty acids.

### **2.15.3 TMS Derivatization**

The sterols were first derivatized for GC analysis. For derivatization, the sterols' hydroxy (OH) group was replaced with a silicon (Si) group (Equation 2.7) (Sigma Aldrich, 2011). Silyl derivatives were more volatile and thermally stable than the sterols (Sigma Aldrich, 2011).



(Equation 2.7)

The hexane from the isolated sterols (Section 2.15.1) was evaporated by nitrogen gas streamed through the test tube while incubated in a water bath. The sterols were re-suspended in 2.0 mL of hexane and a 0.5 mL sample of the solution was transferred to a new clean 10 mL round bottom test tube. The hexane solvent was evaporated as described above. Once all the solvent was evaporated, 100 µL of Tri-Sil BP (BSA:pyridine) (Thermo Fisher Scientific, Canada) was added to the sterols. The test tubes were flushed with nitrogen gas for 10 seconds and the lids were sealed. The test tube was incubated at 68 °C for 20 minutes for the derivatization to take place. After incubation, nitrogen was steamed through the test tubes while being incubated in a water bath at 32 °C until the solvent evaporated. This step lasted approximately 30 minutes to ensure all pyridine was evaporated. The derivatized sterols were re-suspended in approximately 3000 µL of hexane. A sample was transferred into GC vials with a 200 µL insert. The GC vials were capped, crimped, and sealed with dura-seal. The prepared samples were stored at – 20 °C until needed.

### **2.15.4 Fatty Acid Methyl Ester (FAME) Derivatization**

To analyse free fatty acids in yeast by GC, the fatty acids were first derivatized into fatty acid methyl esters (FAME). The solvent was evaporated from the fatty acids with the nitrogen evaporator and the water bath at 32 °C. Once all solvent was

evaporated, 1.5 mL of methylene chloride with 0.01 % w/v BHT and 3.0 mL of the Hilditch reagent was added. The Hilditch reagent was prepared by pouring 100 mL of methanol through filter paper containing approximately 10 g of sodium sulphate anhydrous into an amber reagent bottle. This step ensured that no water was present in the reagent. Additionally, 1.5 mL of concentrated sulphuric acid ( $\text{H}_2\text{SO}_4$ ) was transferred into the amber bottle. The Hilditch reagent was stored at room temperature for no longer than a week.

After the reagents were added, the test tube was flushed with nitrogen. A Teflon lined lid was placed on the test tube, and the sample was mixed by using a vortex mixer for 10 seconds. The samples were incubated on a heating block at 100 °C for 1 h. After the hour, the samples were removed and kept at room temperature for 10 minutes to cool. Distilled water (1 mL) and hexane (3 mL) were added to the test tube. The tube solution was mixed and left to rest until the organic and aqueous layer formed. The organic layer was removed to a new test tube. A 1 mL aliquot of hexane was transferred into the original test tube and the organic layer, which contained the FAME, was transferred to the new test tube. This procedure was repeated one more time. After the hexane was collected the last time, 2 mL of distilled water was added to the test tube that contained FAME in hexane. The test tube was capped and centrifuged for 10 minutes at ½ speed. After centrifugation, the hexane layer was transferred to a new test tube that contained approximately 2 g of sodium sulphate anhydrous. The test tube solution was mixed and the hexane layer was transferred to a pre-weighed test tube. Care was taken at this step to ensure no sodium sulphate crystals were transferred. Once the hexane layer was transferred, the hexane was evaporated with the nitrogen evaporator. The test tube was weighed once all the hexane was evaporated. The FAME was re-suspended in hexane to a concentration of approximately 1mg/mL. The sample was transferred into a GC vial, capped, and sealed with dura-seal until ran on the GC. The vials were stored at – 20 °C until needed.

#### **2.15.5 Sterol Analysis by GC**

Derivatized sterols were analysed by GC-FID to separate sterols and squalene. The GC was fitted with a ZB 3S HT diphenyl dimethyl polysiloxane column (30 m, 0.25 mm I.D., 0.25 µm film thickness) (Agilent Technologies, Palo Alto, USA). A 1 µL injection volume was used with splitless injection with injector held at 250 °C. The initial oven temperature of 60 °C was held for 2 minutes after injection. This was followed by a temperature ramp at 30 °C/minute

to 150 °C. Once reached, the temperature ramp was reduced to 15 °C/minute until 320 °C was achieved. This temperature was held for 10.67 minutes. The carrier gas was helium with a 0.90 mL/minute flow rate. The sterols were identified through GC-MS and library matching using the same parameters for GC analysis. For MS analysis, the ionization energy was 70 eV, with multiplier voltage of 1643 V, source temperature at 200 °C, and transfer line was kept at 250 °C. Spectral data were acquired over a mass range of  $m/z$  60–450.

#### **2.15.6 Fatty Acid Analysis by GC**

The fatty acid methyl esters were analysed by GC-FID. The GC was fitted with a polar DB 23 column (30 m, 0.25 mm I.D., 0.25 µm film thickness) (Agilent Technologies, Palo Alto, USA). A 1 µL injection volume was used with a splitless injector held at 250 °C. The initial temperature of 60 °C was held for 0.50 minutes after injection. The temperature was increased at 45.0 °C/minute to 150 °C. This temperature was held for 2.00 minutes. Last, the temperature was increased at 5.1 °C/minute until 200 °C was reached. This temperature was held for 5.00 minutes. The carrier gas was helium with a 1 mL/minute flow rate. FAME were identified by comparison of retention times with standards.



## Chapter 3 – Industrial Serial Re-Pitched Lager Fermentations

### 3.1 Introduction

In the brewing industry, serial re-pitched lager fermentations are typically re-pitched between 2 and 20 times (O'Connor-Cox, 1997; Powell *et al.*, 2003; Stewart, 2009; Bühligen *et al.*, 2013). However, in one published study with lager yeast, it was determined that one strain had been re-pitched 135 times (Powell and Diacetis, 2007). The impact of serial re-pitching on lager yeast and fermentation has been extensively researched (Moonjai *et al.*, 2003; Speers *et al.*, 2006; Kordialik-Bogacka and Diowksz, 2013; Miller *et al.*, 2013; Deželak *et al.*, 2014). The previously published literature includes the effect that serial re-pitching with lager yeast has on yeasts' genetics (Powell and Diacetis, 2007; Jenkins *et al.*, 2009; Powell and Fischborn, 2010; Lawrence *et al.*, 2012; Bühligen *et al.*, 2013), cell aging (Bühligen *et al.*, 2014), viability and vitality (Jenkins *et al.*, 2003), and ability to flocculate (Smart and Whisker, 1996). Additionally, more research has been undertaken on flavour volatiles produced (Kordialik-Bogacka and Diowksz, 2013; Deželak *et al.*, 2015b), and beer polypeptide profiles (Vieira *et al.*, 2012) between serial re-pitched lager fermentations. Throughout the multiple rounds of serial re-pitching, achieving consistency in beer quality at the end of fermentation is essential from batch to batch. If serial re-pitching leads to an inconsistent product, the process would not be sustainable.

Some Scottish breweries re-pitch the same yeast culture into different worts to produce various brands of beer. The breweries practice this method when the demand for some brands of beer are low, and therefore, they are not brewed each week (B. Smith, *pers. comm.* October 14<sup>th</sup>, 2014; A. Cockburn, *pers. comm.*, November 12<sup>th</sup>, 2012). This re-pitching method creates a chaotic environment for the yeast culture, because it forces the yeast to adapt to changing conditions on top of the already dynamic serial re-pitching environment. Changing the wort type creates variation in original wort densities, hop regimes, carbohydrate levels, and other nutrients. When the malt types and amounts change, the wort carbohydrate profile is influenced (He *et al.*, 2014). Engan (1971) found that the levels of some esters and higher alcohols produced during fermentation varied depending on the carbohydrate type. Additionally, different amino acid concentrations in the wort may affect the levels of some flavour compounds produced during fermentation (Engan, 1970). The pathway for higher alcohol biosynthesis commences with amino acids. A single amino acid leads to the production of a specific higher alcohol (i.e. isoamyl alcohol is produced from leucine) (Pires *et al.*,

2014). Variation in malting, mashing, and boiling affects amino acid compositions in the wort (Jones and Pierce, 1967; Nie *et al.*, 2010).

The study aimed to determine whether re-pitching yeast into worts of different compositions, within one series of re-pitched fermentations, was sustainable. Trends in flavour compound profiles at the end of fermentation were analysed to determine if the profiles remained consistent for each brand of beer produced. In addition, the density attenuation and yeast in suspension profiles were modelled for each fermentation. It was hypothesized that when approximately 5 % of the malt composition changed, the flavour compound levels post fermentation would significantly change ( $p < 0.05$ ).

### **3.2 Experimental Design**

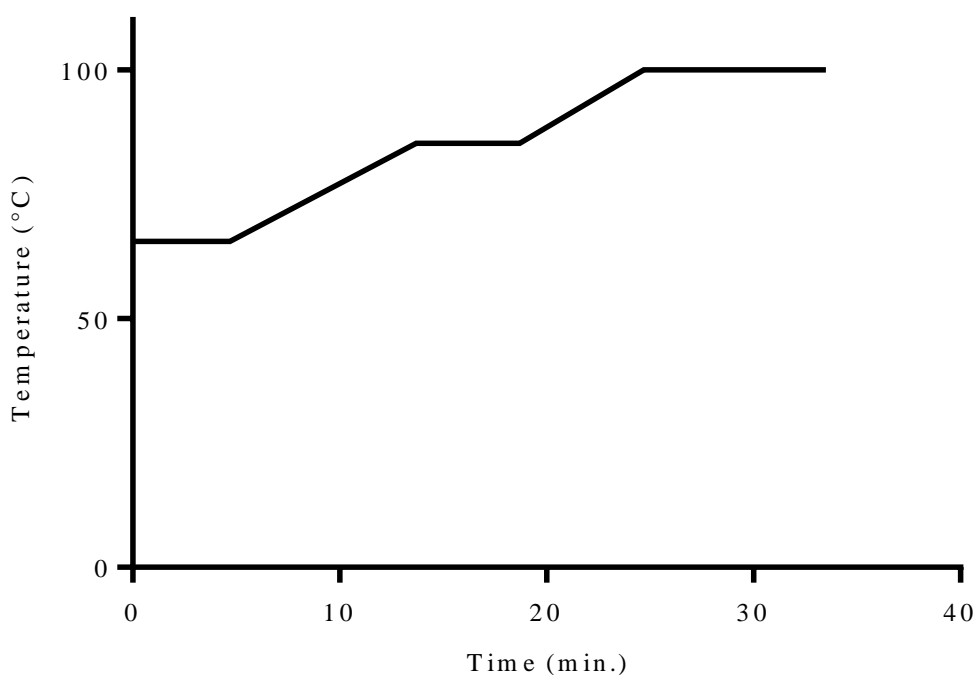
Sufficient yeast for one 1.6 hL fermentation was collected from a large industrial brewery. This was normal practice because the brewery did not have the infrastructure for yeast propagation. This yeast culture was propagated at the industrial brewery and was re-pitched three times prior to collection for this study. The yeast was recovered from the cone of the third fermentation and transferred into a stainless-steel Cornelius keg. The Cornelius keg was cleaned prior to use with 2 % peracetic acid (Murphy and Sons Ltd., Nottingham, UK). The yeast was stored for two days at 11 °C until pitched into the first fermentation for this study, which was considered fermentation one. The yeast was stored at 11 °C as this was the yeast storage temperature used between fermentations. This temperature was chosen because the storage time between re-pitched fermentations was only 6 hours. Even though by reducing the temperature further the yeast's metabolic activity and storage carbohydrates could be reserved, the yeast would also experience cold shock. It was decided that for this short time period, the benefits of reducing the temperature to the ideal 1 – 4 °C storage temperatures did not outweigh the consequences.

A volume of 1.6 hL of wort was prepared prior to each fermentation on the 2 hL pilot brewery at the International Centre for Brewing & Distilling (installed by Briggs, Burton on Trent, UK). There were some small uncontrollable variations in grist composition across the experimental work (Table 3.1).

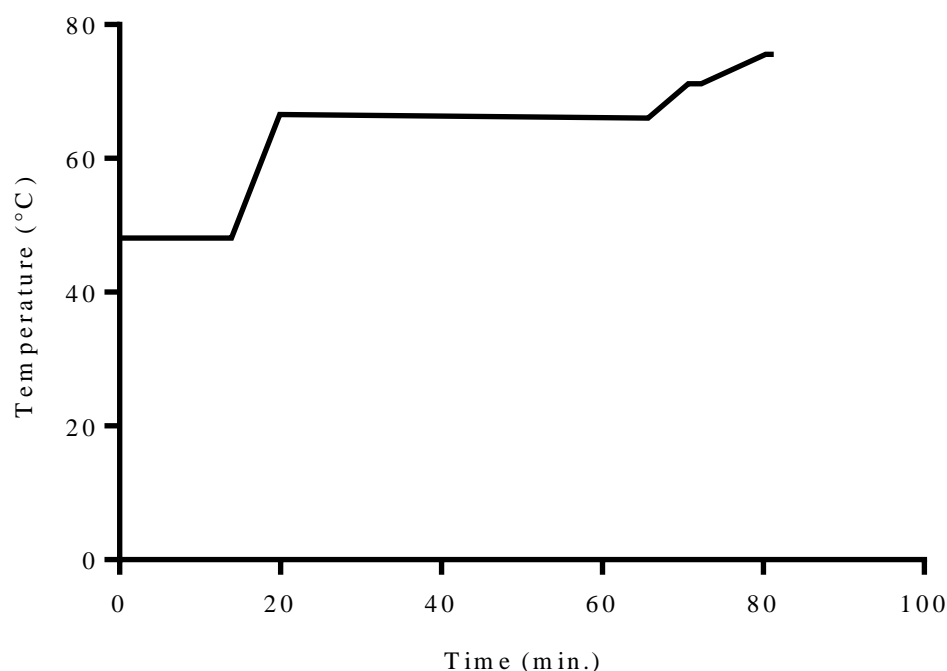
**Table 3.1.** Malt bill used for each fermentation in the serial re-pitching study

<b>Re-Pitched Number</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>
<b>Malt Bill (kg)</b>									
Lager Malt	18.7	18.7	18.7	17.9	24.6	24	17.9	18.7	18.7
Crystal Malt	0.59	0.59	0.59	0	0	1.62	0	0.59	0.59
Munich Malt	0	0	0	1.3	1.62	0	1.3	0	0
Rice	6.15	6.15	6.15	6.15	6.15	6.15	6.15	6.15	6.15

The mashing method used was similar to double mashing, described in section 1.2.2. In the cereal cooker, 6.16 kg of rice and 0.60 kg of lager malt were cooked a liquor to grist ratio of 6 : 1. The temperature started at  $65.5 \pm 2.0$  °C and was held for  $4.7 \pm 1.0$  minutes. The temperature was then increased to  $85.2 \pm 0.3$  °C and held for exactly 5 minutes. Then, the temperature was increased to 100.0 °C and held for  $8.8 \pm 2.3$  minutes (Figure 3.1).

**Figure 3.1.** The temperature profile for the cereal cooker using rice and lager malt.

The liquor to grist ratio for the mash was 2.5 : 1. The initial temperature was  $48.1 \pm 0.9$  °C and was held for  $13.9 \pm 2.2$  minutes. The temperature was then increased to  $66.6 \pm 0.4$  °C and held for  $46.1 \pm 2.2$  minutes. The temperature was then increased to  $71.2 \pm 0.2$  °C and held for  $1.6 \pm 0.9$  minutes. Finally, the temperature was increased to  $75.6 \pm 0.4$  °C and held for exactly 1 min (Figure 3.2).



**Figure 3.2.** The temperature profile for the mash tun using lager malt and either Crystal malt or Munich malt.

The solid liquid separation was achieved by lauter tun and sparged as necessary. First the wort was recirculated until clear, and then transferred to the kettle. The collected wort was boiled for one hour. At the start of boil, 41.6 g of pelleted Admiral hops (John I Hass Inc., Washington DC, USA) were added, and at the end of the boil, 160 g of pelleted First Gold hops (John I Hass Inc., Washington DC, USA) were added to achieve approximately 10 IBU. After the wort was boiled, the wort was cooled using a plate heat exchanger and transferred to a 2 hL cylindroconical fermenter.

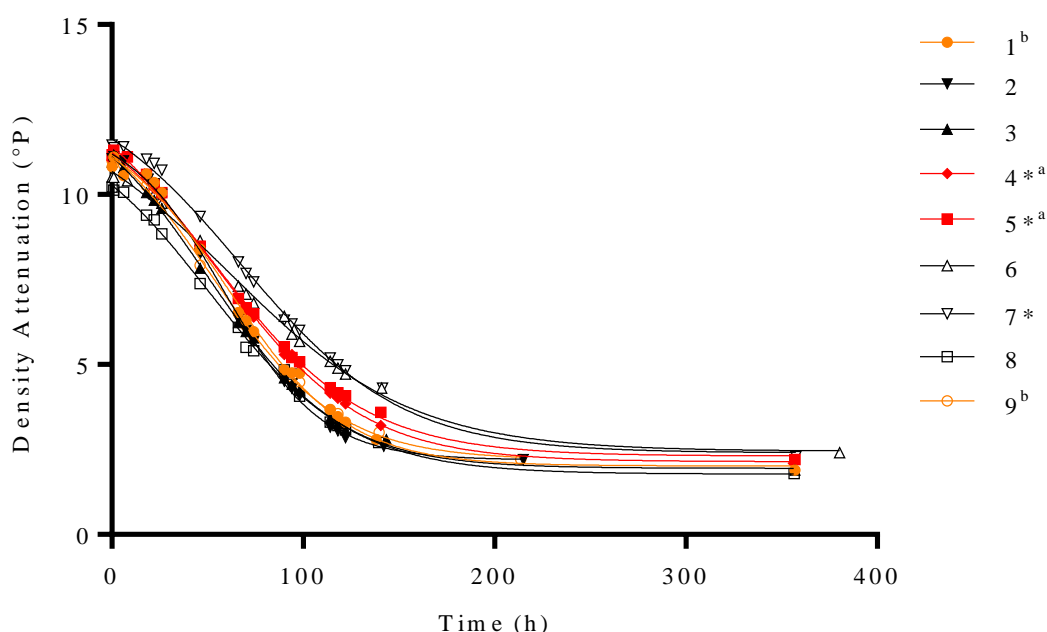
The yeast was counted and viability checked (Section 2.5.2) and pitched at  $1.1 \times 10^7$  viable cells/mL by volume into the fermenter with the prepared wort. The fermentations lasted six days and were set at 16 °C, following which, the temperature was set to cool to 1 °C. Each fermentation was set to cool after 138 hours  $\pm$  1 hour regardless of the density achieved, allowing the collection of yeast for the subsequent fermentation. The fermenter was cooled for 24 hours, which allowed yeasts to sediment in the cone and were collected before the next fermentation. Between fermentations, yeast was stored for six hours at 11 °C. This process was repeated nine times for a total of nine fermentations utilizing the same yeast culture. During the fermentation, density and absorbance measurements were taken, and the data was subjected to mathematical modelling (Section 2.3.3). At the end of fermentation at hour 138, a 45 mL sample was

frozen in a 50 mL centrifuge tube and analysed later for flavour volatile compounds (Section 2.9). Linear regression was performed on the flavour volatile compounds to determine if there were linear trends related to the crop number. For this chapter,  $p < 0.05$  was considered significant for all tests.

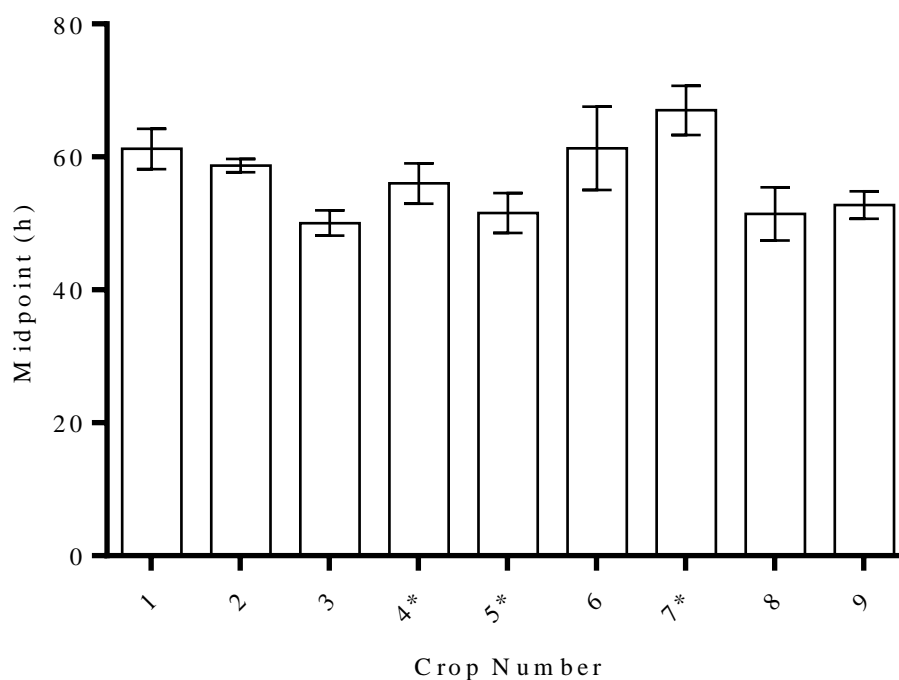
### 3.3 Results

#### 3.3.1 Physical Fermentation

The density for all nine serial re-pitching fermentations started between 10.22 and 11.49 °P. By approximately hour 357, the density had decreased to between 1.8 and 2.4 °P. The density attenuation was modelled using the four-parameter non-linear regression (Section 2.3.3) for nine serial re-pitched fermentations (Figure 3.3). Fermentations four and five and fermentations one and nine were not significantly different. In the four-parameter regression, parameter “M” signified the midpoint of the fermentation. The midpoint between each of the nine serial re-pitched fermentations was found to vary between hour 50 and 62 (Figure 3.4).



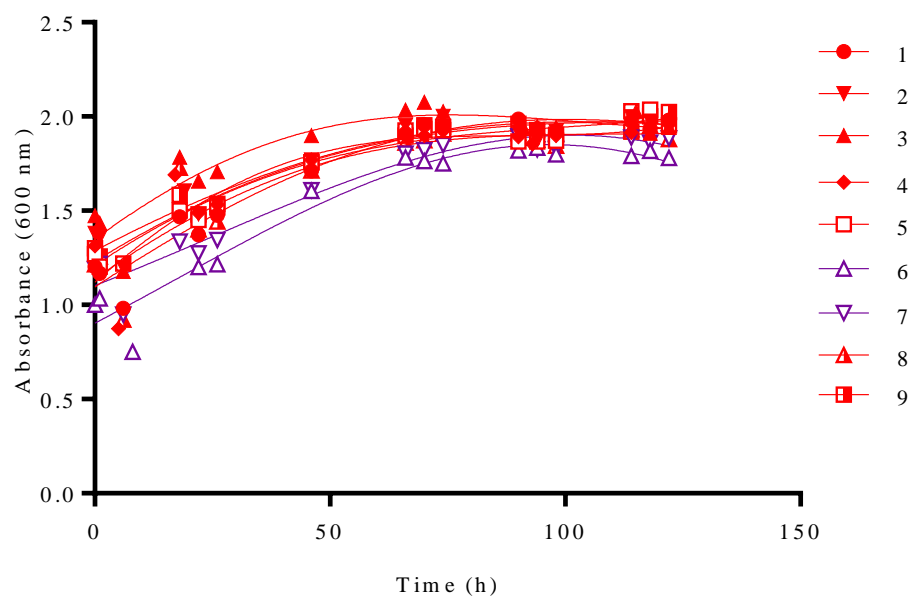
**Figure 3.3.** Density attenuation for nine serial re-pitched 1.6 hL lager fermentations. The F-Test showed that the regression for fermentations one and nine (<sup>b</sup>/orange) and fermentations four and five (<sup>a</sup>/red) were not significantly different. The fermentations marked “\*” contained Munich malt and the fermentations without contained Crystal malt.



**Figure 3.4.** The midpoint from the modelled density attenuation as the yeast crop number increased. The crop numbers indicated by “\*” contained with Munich malt in the malt bill and the remaining crop numbers contained Crystal malt. Error bars indicated the standard error of the mean from the calculated nonlinear regression.

Application of a one-way ANOVA showed that at least one midpoint was significantly different ( $F = 2.925$ ,  $p = 0.0046$ ). Following this test, Tukey’s Multiple Comparisons test showed that the crop seven midpoint was significantly different from three, five, and eight. The midpoints for all other crop numbers showed no significant differences.

The absorbance (600 nm) was also measured throughout all nine serial re-pitched fermentations (Figure 3.5). The absorbance was measured for all fermentations to compare yeast in suspension measurements relative to the other fermentations. This was chosen instead of cell counts due to the high frequency of the measurements. This method allowed a quick estimate of the cells in suspension. This was desired to the cell counts because cell counts could become laborious and a precise measurement of the cells in suspension were not required. The absorbance for all fermentations started between 1.002 – 1.474. The absorbance for all fermentations increased at the beginning of fermentation, and then appeared to reach a plateau. There were no observable trends in changes of absorbance over the course of the fermentation and the increasing yeast crop number.



**Figure 3.5.** Yeast in suspension trends over nine serial re-pitched fermentations measured by the absorbance at 600nm. Yeast in suspension models marked in red were not significantly different. The models marked in purple were also not significantly different.

At the start of the fermentation, the yeast was counted and the viability was assessed for pitching (Table 3.2). In the first fermentation, the % viable cells in the culture was  $97.11 \pm 0.50$  %. As the yeasts were re-pitched, the % viable cells remained between 94.39 and 96.44 %, except for fermentation eight, which had the lowest viability at  $92.85 \pm 3.54$  %.

**Table 3.2.** The average % viable cells in the yeast culture of duplicate measurements

Re-Pitched Ferm.	% Viable (Avg.)	SD
1	97.11	0.50
2	94.83	0.91
3	96.24	0.97
4	96.44	0.25
5	94.39	1.58
6	95.25	0.69
7	95.59	0.37
8	92.85	3.54
9	95.83	1.55

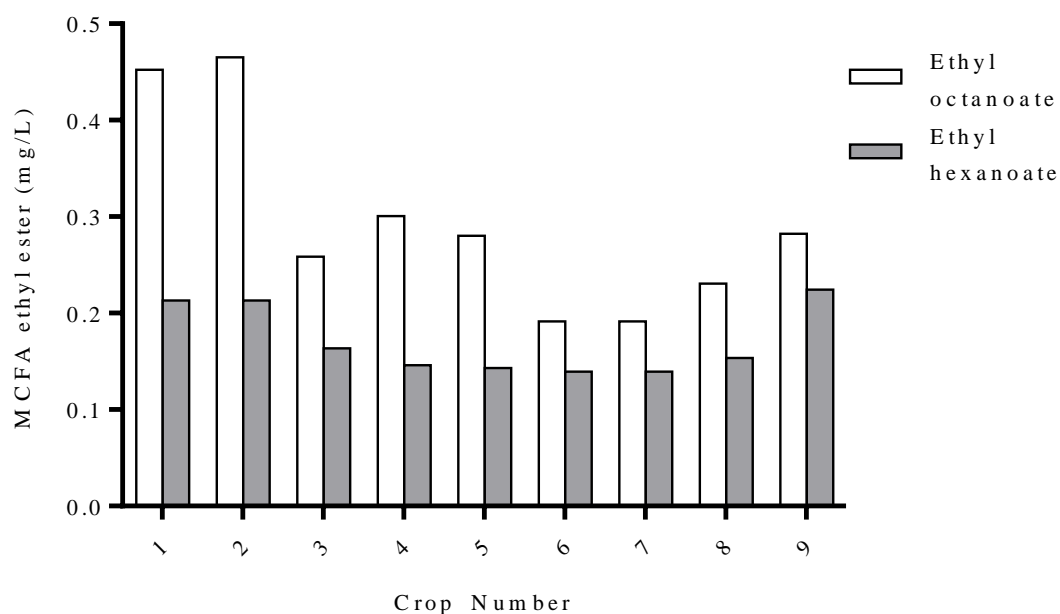
### 3.3.2 Flavour Profile

Samples for volatile flavour compound analysis were collected at hour 138 of each fermentation. The MCFA ethyl esters analysed were ethyl octanoate and ethyl hexanoate (Figure 3.6). Ethyl octanoate levels were high, between 0.45 and 0.46 mg/L, for the first two fermentations and the proceeding fermentations remained between 0.19 and 0.30 mg/L. Ethyl hexanoate levels were between 0.14 and 0.22 mg/L for all nine serial re-pitched fermentations.

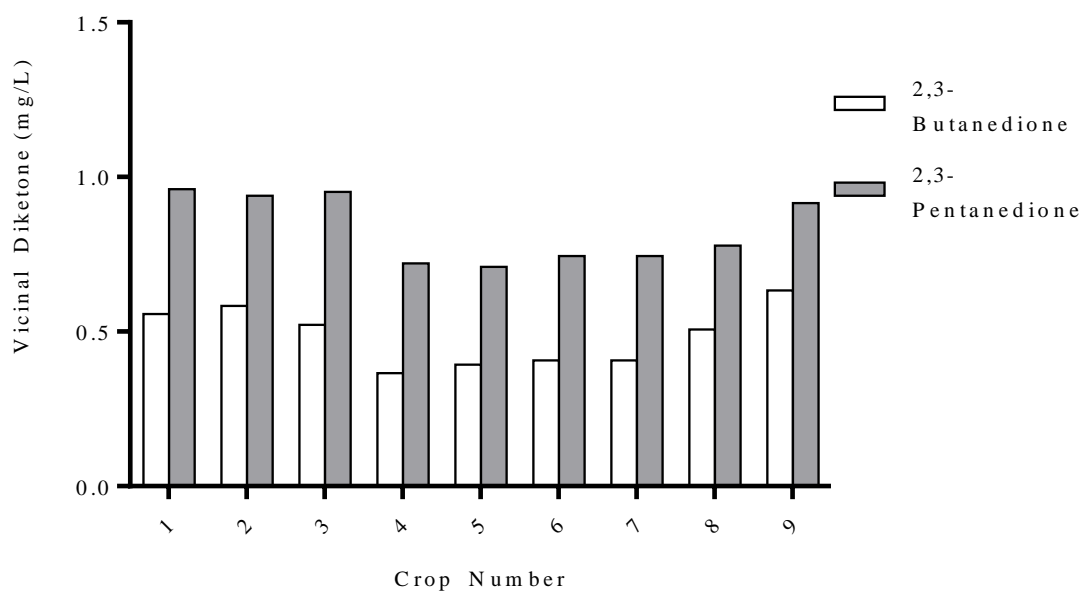
The vicinal diketones analysed were 2,3-butanedione and 2,3-pentanedione. These levels post fermentations were approximately 0.5 mg/L of 2,3-butanedione and 0.9 mg/L 2,3-pentanedione for the first three fermentations (Figure 3.7). In the fourth fermentation, the levels declined to approximately 0.3 and 0.7 mg/L respectively and following this increased until the ninth fermentation up to 0.6 mg/L of 2,3-butanedione and 0.9 mg/L of 2,3-pentanedione.

Propan-1-ol levels remained between 9 and 11 mg/L for all fermentations and, isobutanol levels were between 6 and 8 mg/L. These levels were below their published flavour thresholds of 600 mg/L and 100 mg/L respectively (Pires and Brányik, 2015). It was determined that 2-methylbutanol levels were between 9 and 13 mg/L and 3-methylbutanol levels demonstrated the largest variation with levels reported between 28 and 40 mg/L (Figure 3.8). These two higher alcohols were also below their published flavour thresholds of 65 and 70 mg/L respectively (Olaniran *et al.*, 2017).

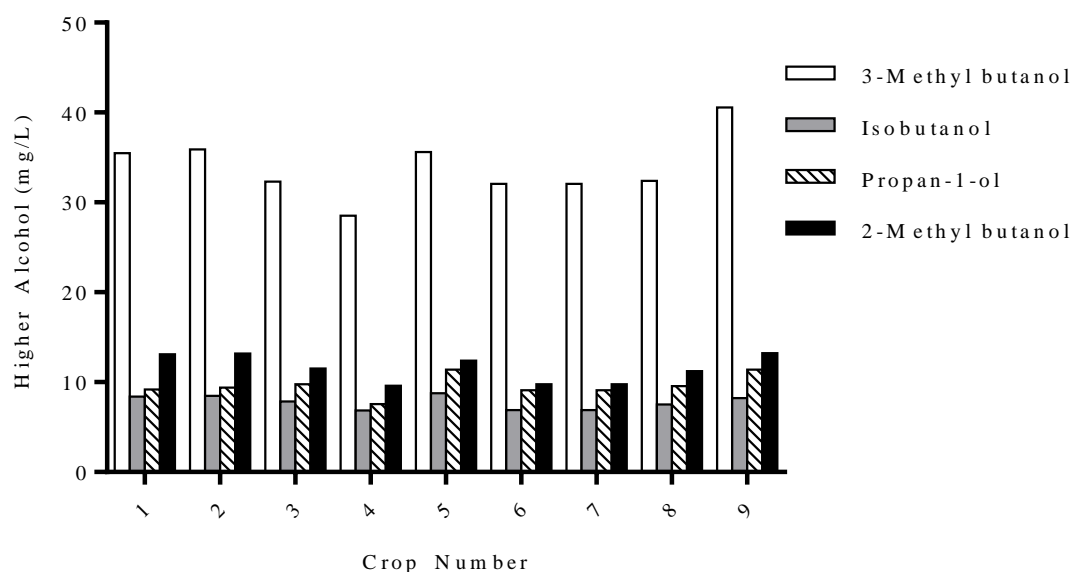




**Figure 3.6.** Levels of MCFA ethyl esters (ethyl octanoate and ethyl hexanoate) present at hour 138 of the fermentation for nine serial re-pitched fermentations.

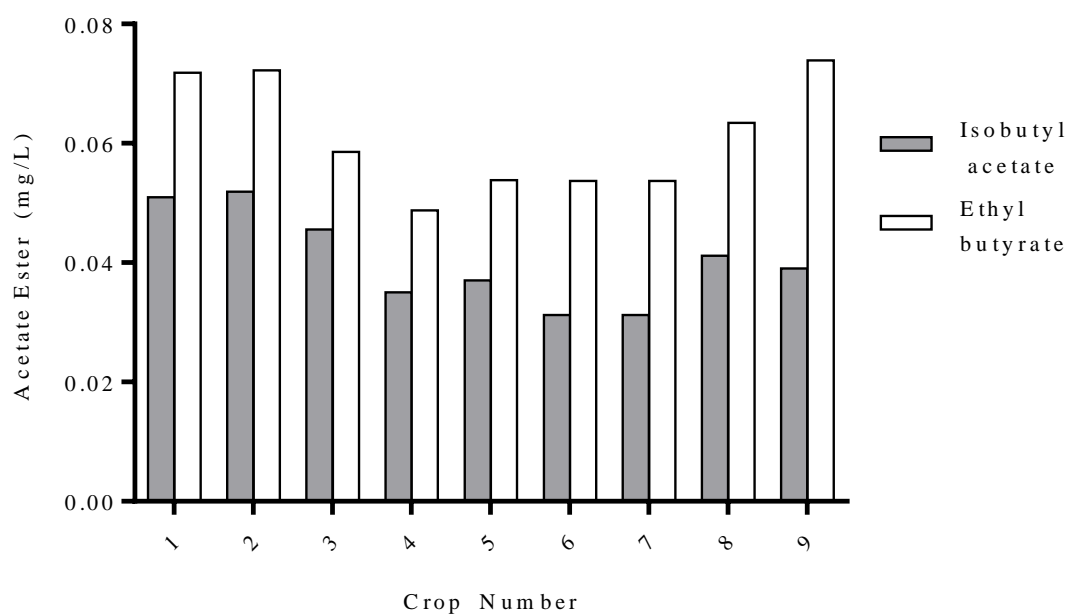


**Figure 3.7.** Levels of vicinal diketones (2,3-butanedione and 2,3-pentanedione) present at hour 138 of the fermentation for nine serial re-pitched fermentations.

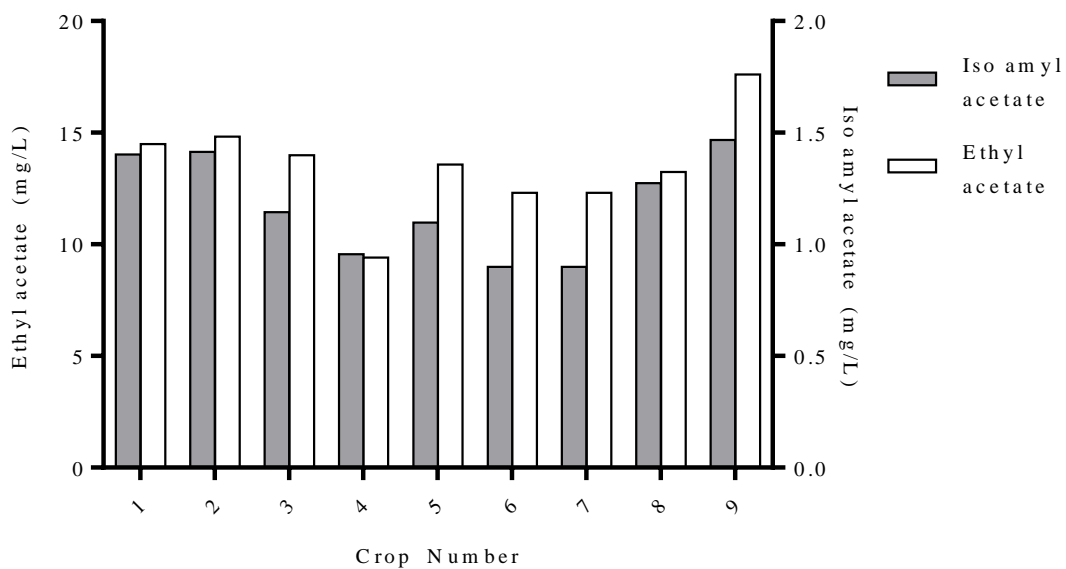


**Figure 3.8.** Levels of higher alcohols (3-methyl butanol, isobutanol, propan-1-ol, and 2-methyl butanol) present at hour 138 of the fermentation for nine serial re-pitched fermentations.

The acetate esters analysed were isobutyl acetate, ethyl butyrate, isoamyl acetate, and ethyl acetate. Levels of isobutyl acetate were between 0.045 and 0.052 mg/L for the first three fermentations (Figure 3.9). The following four fermentations contained lower levels of isobutyl acetate, between 0.031 and 0.037 mg/L. The last two fermentations, 8 and 9, had levels between 0.39 and 0.41mg/L. The levels of ethyl butyrate were highest in fermentations one, two, and nine, with a concentration between 0.072 and 0.074 mg/L (Figure 3.9). Fermentations three, four, five, six, seven, and eight, contained lower levels of ethyl butyrate between 0.048 and 0.063 mg/L. Isoamyl acetate levels after fermentations one and two were 1.40 – 1.41 mg/L. Then the levels decreased and remained at a similar concentration for fermentations three, four, five, six, and seven at approximately 0.89 – 1.14 mg/L (Figure 3.10). The last two fermentations contained higher levels than the previous fermentations with 1.27 – 1.46 mg/L of isoamyl acetate. Finally, the levels of ethyl acetate were between 12.31 and 14.82 mg/L for fermentations one, two, three, five, six, seven, and eight (Figure 3.10). Fermentation four had a lower concentration of ethyl acetate at 9.41 mg/L and fermentation nine had a higher concentration at 17.61 mg/L.



**Figure 3.9.** Levels of two acetate esters (isobutyl acetate and ethyl butyrate) present at hour 138 of the fermentation for 9 serial re-pitched fermentations.



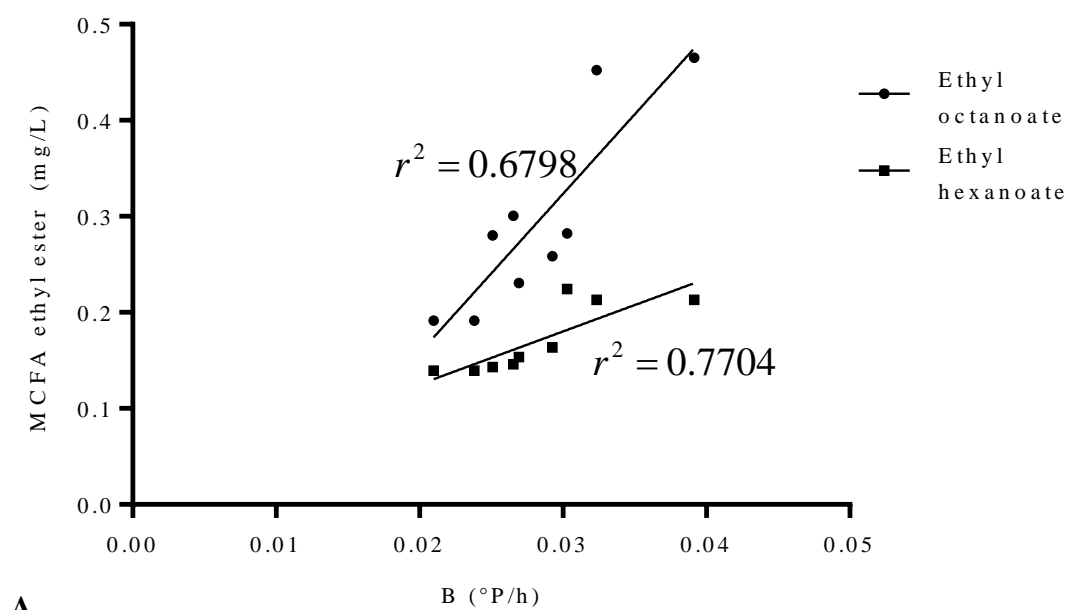
**Figure 3.10.** Levels of two acetate esters (ethyl acetate and isoamyl acetate) present at hour 138 of the fermentation for 9 serial re-pitched fermentations.

For each flavour compound analysed, one sample was obtained per fermentation. Linear regression was utilized to determine if linear trends in concentrations flavour compounds existed with an increasing crop number. Ethyl octanoate was the only flavour compound that showed a significant linear relation to the crop number in a decreasing order, but there was a large degree of variation ( $r^2 = 0.5441$ ). In addition to the linear regression analysis, the flavour profiles based on the malt compositions were statistically analysed by a Student's T-test (Table 3.3). The analysis suggests that the malt composition had a significant effect on three flavour compounds. The levels of ethyl butyrate, 2,3-butanedione and 2,3-pentanedione at the end of fermentation were higher when part of the wort composition contained Crystal malt as opposed to Munich malt.

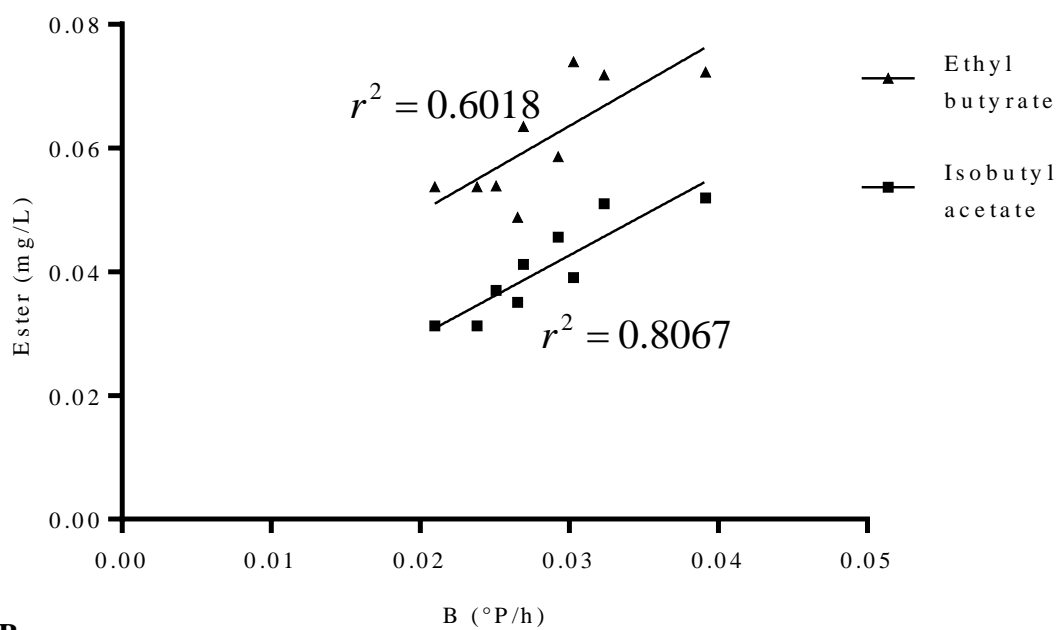
**Table 3.3.** Comparison using a T-test to determine if differences exist between the fermentations with Munich malt and Crystal malt for the indicated flavour compound.

Flavour Compound	p Value	Level of Significance
Esters		
Ethyl acetate	0.0909	ns
Isobutyl acetate	0.1068	ns
Ethyl butyrate	0.033	*
Isoamyl acetate	0.0727	ns
Ethyl hexanoate	0.098	ns
Ethyl octanoate	0.4678	ns
Higher Alcohols		
Propan-1-ol	0.6869	ns
Isobutanol	0.4933	ns
2-Methyl butanol	0.2148	ns
3-Methyl butanol	0.2904	ns
Vicinal Diketones		
2,3-Butanedione	0.0167	*
2,3-Pentanedione	0.0288	*
ns = not significant, * = $p < 0.05$ , ** = $p < 0.01$		

There were no consistent trends found between the levels of flavour compounds post fermentation and the crop number, with the exception of ethyl octanoate. However, the levels of isoamyl acetate, 3-methyl butanol, ethyl hexanoate, ethyl octanoate, 2,3-butanedione and 2,3-pentanedione post fermentation had positive linear correlations to the parameter B in the modelled density attenuation. The parameter B represented the rate of sugar consumption at the midpoint of the fermentation. Ethyl hexanoate, ethyl octanoate, ethyl butyrate, and isobutyl acetate are shown (Figure 3.11).



**A**



**B**

**Figure 3.11.** Ethyl octanoate and ethyl hexanoate (A) and ethyl butyrate and isobutyl acetate (B) correlated to the parameter “B” on the modelled the density attenuation during fermentation.

### **3.4 Discussion**

#### **3.4.1 Density Attenuation**

The density attenuation was modelled to determine whether there were differences between (1) the crop number or (2) the differing malt compositions. The fermentations with different malt compositions did not exhibit the same density attenuation profiles. Fermentations four, five, and seven (which contained Munich malt) were all significantly different from fermentations one, two, three, seven, eight, and nine (which contained Crystal malt). With the differing malt compositions, the carbohydrate make-up of the wort changed. Previous studies have demonstrated that the carbohydrate composition effects the fermentation rates (Dekoninck *et al.*, 2012) and therefore, this observation was as expected. However, there were also multiple instances where density attenuation rates between fermentation with the same malt composition were significantly different. For example, fermentations two and three were statistically different and those fermentations contained the same malt composition. This difference found in fermentation trends could not be related to the % viability of the yeast culture, because their viabilities were not statistically different. A couple factors may have influenced the differences observed between density attenuation trends with the same malt and difference yeast generation such as the wort oxygenation levels. During this study, the DO levels were not controlled. The generation of the yeast, however, cannot be eliminated as a cause even though the differences observed between fermentation two and three are minimal.

There were instances where two density attenuation trends were not significantly different. This was the case for the first and the last fermentation in the serial re-pitching process. This was an indicator that there was no overall increasing or decreasing change in sugar consumption rates when re-using the same yeast culture. Even with the disruption of the changed malt composition within the serial re-pitching cycle, the density attenuation rate for fermentation nine exhibited identical attenuation rates as an earlier fermentation. When there was a 2.32 – 5.13 % (w/w) change of malt composition within the re-pitching cycle, it was found that the overall fermentative ability stayed conserved when returned to a fermentation with media matching the original malt bill.

#### **3.4.2 Yeast in Suspension**

The method utilized to measure the yeast in suspension was from ASBC Yeast-14 by absorbance at 600 nm. This monitored the increase in absorbance as yeast growth took place during fermentation and the decline in absorbance as the yeast flocculated towards the end of fermentation. In the current study, a decrease in absorbance was not

apparent towards the end of fermentation. It is suggested that this was likely to be caused by the sampling location during fermentation. The sample port on the tank was located at the bottom third (by height) of the tank. This meant that flocculated yeast was likely to be collected along with the yeast in suspension and thus altered the yeast flocculation trends observed towards the end of fermentation. The growth rates, determined by increase in absorbance, were 1.2 – 1.8 times the original absorbance. These values, however, are solely relative because the turbidity of unpitched wort was unknown so the actual yeast growth during fermentation could not be determined. Given that, there was enough growth during fermentation for adequate yeast to be collected at the end for re-pitching.

### **3.4.3 Flavour Profiles**

#### *Higher Alcohols*

It was hypothesized that significant differences between the levels of higher alcohols between fermentations with Munich malt and fermentation with Crystal malt would exist. Surprisingly, this was not found (Table 3.2). No levels of higher alcohols examined were significantly different between the two malt compositions. A new hypothesis was formed that the small differences in malt compositions were not large enough to make a difference on the higher alcohol concentrations produced. Munich or Crystal malt made up between 2.32 – 5.13 % (w/w) of the malt bill, and the remaining 94.87 – 97.68 % remained consistent throughout all the fermentations.

#### *Esters*

The first two fermentations were found to have increased concentrations of ethyl octanoate. The levels of ethyl octanoate in the subsequent fermentations remained static as the crop number increased. The original yeast culture utilized for this experiment was collected and stored for two days prior to commencing the fermentations. The longer storage time may have contributed to the increased levels of ethyl octanoate. A previously published study demonstrated that some strains of wine yeasts, when exposed to different environmental conditions such as hyperosmotic stress, some flavour compounds were impacted (Fairbairn *et al.*, 2014). For this study, it is possible that the longer exposure to high ethanol conditions affected the cells production of ethyl octanoate. Little literature exists that demonstrates why esters are produced, so further studies would need to be completed to investigate this.

No other flavour compound analysed varied with the crop number. This demonstrated no overall trend with the crop number that impacted the beer flavour

profile. These results agreed with a previously published study that found no significant variation in the beer flavour profile over ten re-pitched fermentations (Kordialik-Bogacka and Diowksz, 2013)

The only trend found with the ester profile of the beers was that four esters were correlated to the parameter B on the nonlinear model. Those four esters were ethyl octanoate, ethyl hexanoate, ethyl butyrate, and isobutyl acetate. The parameter B represents the quickest rate of sugar consumption, which takes place at the midpoint of the fermentation. The rates of ester formations are the highest during the most vigorous point of fermentation (Yoshioka and Hashimoto, 1983; Pires *et al.*, 2014). The most vigorous point of fermentation is the midpoint of the fermentation, so the observation of this correlation was unsurprising.

#### *Vicinal Diketones*

Levels of both 2,3-butanedione and 2,3-pentanedione were significantly affected by the malt composition variation. The approximately 5 % change in malt bill had a significant change in the levels post fermentation. The difference in Crystal and Munich malt significantly affected the levels of vicinal diketones in these fermentations. The concentrations of 2,3-butanedione and 2,3-pentanedione were lower after fermentations completed that contained Munich malt when compared to fermentations that contained Crystal malt. Variation in malting can change the amino acid levels in the malted barley (Nie *et al.*, 2010). Additionally, the amino acid valine in the wort is correlated to the concentration of 2,3-butanedione post fermentation (Portno, 1966). An assumption could be made that the significant differences observed were related to differing amino acid concentrations. Isobutanol concentrations, however, are also correlated to valine concentrations in the wort (Pires *et al.*, 2014). If the amino acid concentrations of the worts impacted 2,3-butanedione levels, it might be hypothesized that isobutanol levels produced should vary as well. Isobutanol levels were unchanged between the differing malt compositions. Therefore, this phenomenon observed is likely more complex than a potential amino acid composition variation.

Overall, the malt composition difference had a significant effect on the levels of 2,3-butanedione, 2,3-pentanedione, and ethyl butyrate. However, the other nine flavour compounds analysed were unaffected. The only flavour compound that seemed to significantly decrease with the crop number was ethyl octanoate. This result, however, may have been related to longer yeast storage times before the start of the re-pitching process.



## Chapter 4 – Industrial Serial Re-Pitched Ale Fermentations

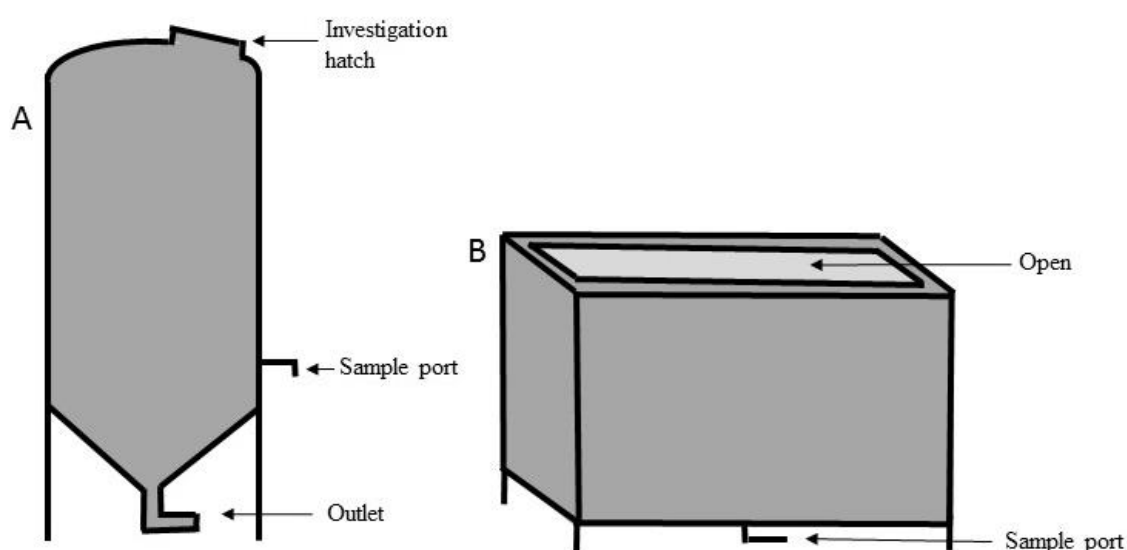
### 4.1 Introduction

Many studies have been undertaken to monitor fermentation changes that occur as yeast cultures are re-pitched with lager strains (Speers *et al.*, 2003; Kobayashi *et al.*, 2007; Powell and Diacetis, 2007; Speers and Stokes, 2009; Powell and Fischborn, 2010; Vieira *et al.*, 2012; Bühligen *et al.*, 2013, 2014; Kordialik-Bogacka and Diowksz, 2013). Serial re-pitching with ale yeast in literature has been the subject of some studies, but is not as prevalent (Smart and Whisker, 1996; Cunningham and Stewart, 2000; Powell and Diacetis, 2007).

One of these studies investigated DNA sequences in ale yeast during the re-pitching (Powell and Diacetis, 2007). No variation was found between the sequences analysed between a fresh ale yeast culture and the same yeast culture that had been re-pitched 98 times. The same study concluded that no differences between fermentation characteristics with the ale yeast strain were observed. The authors suggested that while genetic drift can occur spontaneously, their data indicated that some strains' genetics might remain conserved during re-pitching (Powell and Diacetis, 2007). Another study investigated ale strain flocculation characteristics during serial re-pitching. Extended serial re-pitching of an ale strain with laboratory scale fermentations showed that the flocculation increased in the beginning of the serial re-pitching process and this characteristic remained conserved until the 24<sup>th</sup> fermentation of serial re-pitching and then the flocculation properties deteriorated in subsequent fermentations (Smart and Whisker, 1996). Another previously published study conducted with ale strain serial re-pitching investigated how different initial oxygenation levels and the impact of acid washing affected fermentation characteristics (Section 1.2.7) (Cunningham and Stewart, 2000). The authors found that sufficient wort oxygenation levels were critical for yeast to successfully survive acid washing between rounds of serial re-pitching (Cunningham and Stewart, 2000).

Ale yeasts have a different flocculation behaviour than lager yeasts. Most notable that ale yeasts are top fermenting (Jelinek, 1946; Bokulich and Bamforth, 2013). As the fermentable carbohydrates become depleted in fermenting wort, the ale yeasts flocculate and the aggregates entrap gaseous CO<sub>2</sub> (Speers *et al.*, 1992a; Dengis *et al.*, 1995). These cells become buoyant and thus rise to the top of the fermenter. As more yeast cells flocculate, a kräusen layer is formed at the surface of the fermenting beer (Jelinek, 1946; Hardwick, 2006). As the fermentation reaches equilibrium, the kräusen

layer is traditionally skimmed from the surface and stored in a yeast storage tank for re-pitching. Historically, ale fermentations took place in open fermenters instead of the CCVs that are typically used today (Figure 4.1). There are breweries that utilize open fermenters today, however they are not as common anymore. CCVs are common in modern breweries as they are efficient, lower cost, and have a smaller foot print (Lodolo *et al.*, 2008). When ale strain fermentations take place in CCVs, the strains that have a higher ability to flocculate will sediment in the cone instead of rising to the top (Speers *et al.*, 1992b; Soares, 2010).



**Figure 4.1.** Simple design of a CCV (left) and an open fermenter (right).

The external factors encountered by flocculated ale yeasts in open fermenters differ from the external factors encountered by flocculated lager yeasts (Table 4.1). The kräusen layer is in contact with the atmosphere, which may cause airborne bacteria to contaminate the yeast. For this reason, acid washing is common, because bacterial contamination may be problematic (Cunningham and Stewart, 2000). Care must be taken when acid washing, because if the initial wort oxygenation level is insufficient, acid washing may negatively impact cell growth during fermentation (Cunningham and Stewart, 2000).

**Table 4.1.** Differing environmental conditions encountered by flocculated ale and lager yeast post primary fermentation.

Factor	Yeast Strain	
	Ale	Lager
Pressure	Atmospheric Pressure	Hydrostatic pressure (dependent on height)
Oxygen	Present	Not Present
Temperature	Room temperature	Possible hot spots formed from packed yeast cells and metabolism
Ethanol	Same percentage as in the fermented beer	Higher than fermented beer as some cells continue to ferment in the cone

No work on serial re-pitching specifically with ale yeast and the flavour profiles could be identified. The flavour profiles of a beer are important when addressing beer quality, because these compounds have very low flavour thresholds (Pires *et al.*, 2014) (Section 1.3.6). The study presented here tests the hypothesis that serial re-pitching ale yeast nine times in open fermenters has no impact on ‘green’ beer quality. This hypothesis was tested through the collection of industrial sampling and data collection from a local brewery owned by a global brewing company. Trends in fermentation properties were examined by comparisons between fermentation density attenuation, carbohydrate levels and production of volatile flavour compounds.

## 4.2 Experimental design

Nine serial re-pitched 213 hL ale fermentations were monitored at a UK brewery. Propagation was started by the brewery by inoculating one sterile loopful of yeast into 2 x 500 mL of their YM broth. The propagation was completed in accordance with the breweries existing standard operating procedure. The yeast was cultured with constant aeration (at an undefined rate) for 24 hours. For the second propagation stage, the cultured yeast was pitched into 2 x 5 L of wort starting at 14.7 °P for approximately 24 hours under constant aeration. Finally, the yeast cultures from the 2 x 5 L propagators were combined and inoculated into 1 x 11 hL wort at 14.7 °P and then aerated for 24 hours. The wort utilized for the second and third stage of propagation was the same wort used for the series of fermentations examined. The brewery would not disclose the exact grist composition of the wort; however, it was produced with Optic and Golden Promise barely malt. The hops used for these fermentations were whole cone Fuggles, Aurora, Savinski Goldings, and Cascade hops to achieve 30 IBU.

Prior to fermentation, the yeast culture was acid washed in a 25 hL tank using phosphoric acid (pH 2.0-2.3) at 4 °C under constant mechanical agitation for two hours. Yeast was pitched directly into the wort line during the wort transfer to a 213 hL fermenter, after 16 – 49 hL of wort was already transferred to the fermenter. The wort was oxygenated prior to the transfer. At the start of fermentation, a sample was taken to determine the achieved pitching rate with an Improved Neubauer haemocytometer (Section 2.5.1). All sampling during fermentation was taken using a metal cylinder with a chain that was rinsed with 2 % peracetic acid. The cylinder was dropped into the top of the fermenter and sunk to 2/3<sup>rd</sup> depth of the vessel. This ensured collection of fermenting wort. Once full, the cylinder was retrieved using the stainless-steel metal chain. The specific gravity was measured by hydrometer at hour 0, 12, and every subsequent four hours until the end of fermentation. To keep the units of density consistent, the specific gravity measurements were converted to density in °P (Equation 4.1) from methods of ASBC Wort-3. The equation (4.1) was used to estimate the density in °P, which is reported to be within  $\pm 0.002$  °P as stated within the ASBC method, Wort-3. This was true for all measurements above 0.5 °P. As the experimental data was below 0.5 °P, this error was not of concern.

$$Plato = 135.997(SG)^3 - 630.272(SG)^2 + 1111.14(SG) - 616.868$$

(Equation 4.1)

Additionally, a 50 mL aliquot of fermenting beer was taken at each sampling time, frozen at -18 °C, and stored until further analysis for yeast in suspension measurements, carbohydrate concentrations analysis, and/or flavour concentrations analysis. The yeast in suspension samples were thawed at room temperature and measured as described in section 2.3.2. The density attenuation and yeast in suspension trends were modelled using nonlinear regression techniques (Section 2.3.3).

At hour 0 and when the fermentation was substantially complete (95 % determined by the nonlinear regression analysis of the density attenuation during fermentation), carbohydrate analysis was completed using High Performance Anion Exchange (HPAE) (Section 2.10) and flavour analysis was performed using Gas Chromatography (GC) (Section 2.9).

The data collected for higher alcohols, esters, and vicinal diketones levels were analysed using linear regression to determine whether the crop number had a significant

impact on the flavour compounds post primary fermentation. The statistical analysis was performed using Graphpad Prism (Graphpad Software, La Jolla, USA). A 90 % significance level was used for the density attenuation rates. This was done because the breweries methods for pitching yeast to start fermentation were not precise. The company pitched the yeast cell slurry by weight, and not cell count. Published literature showed that the pitching rate affects density attenuation rates and the production of certain flavour compounds during fermentation (Erten *et al.*, 2007). For this reason, the 95 % significance level was relaxed to 90 % significance in attempt determine if the crop number affected density attenuation rates even with this variation.

Upon fermentation completion, the fermenter was set to cool once the density reached between 2.6 and 3.3 °P. This was between hour 40 – 88 depending on the fermentation. When the tank was set to cool, the kräusen was collected into a 33 hL yeast collection tank and then transferred to a 49 hL yeast storage tank. The yeasts were stored until needed for fermentations the following week. Immediately prior to re-pitching, the cells were acid washed. As per company protocol, multiple brands of beer utilized the same re-pitched yeast culture. Once each brand of fermentation was completed, the skimmed yeast was combined into one yeast storage tank. At the start of the week, all fermentations were pitched and therefore all yeasts were considered to be of the same generation, however yeast storage times of the yeast within this vessel varied from 8 to 195 hours due to uneven fermentation times.

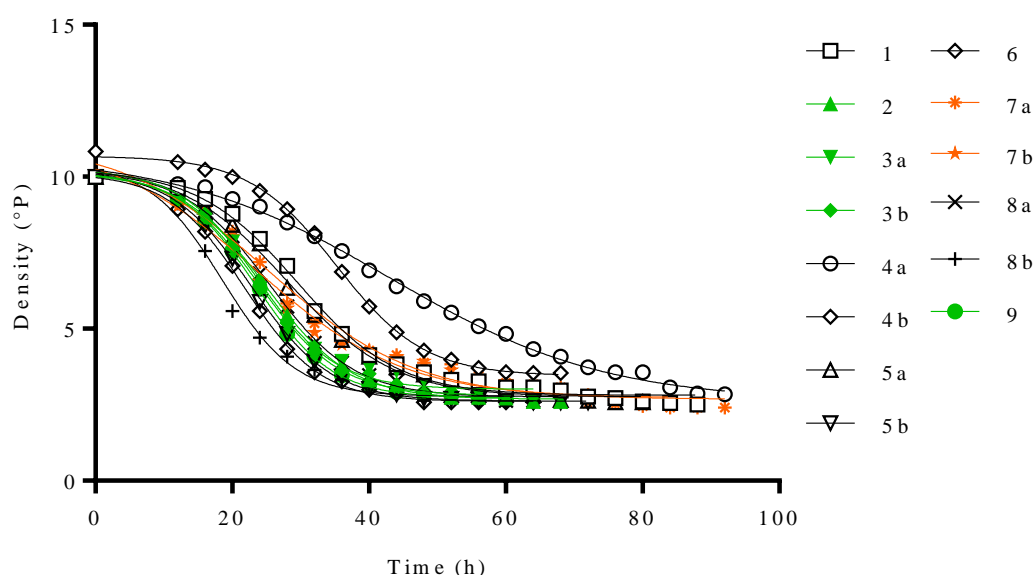
The yeast culture was serial re-pitched nine times. Serial re-pitched fermentations three, four, five, seven, and eight, were completed in duplicate for the fermentations because enough yeast was present for two fermentations. Serial re-pitched fermentations one, two, six, and nine only had sufficient yeast mass to re-pitch one fermentation.

## **4.3 Results**

### **4.3.1 Density Attenuation Analysis**

The wort's density was monitored throughout all nine serial re-pitched fermentations, and re-pitched fermentations three, four, five, seven, and eight were completed in duplicate (Figure 4.2). An F-Test was used to confirm that crop numbers two, three (a), three (b), and nine showed no significant difference between the fermentation density profile ( $p > 0.1$ ). Independently, re-pitched fermentations seven (a) and seven (b) showed no significant difference ( $p > 0.1$ ).

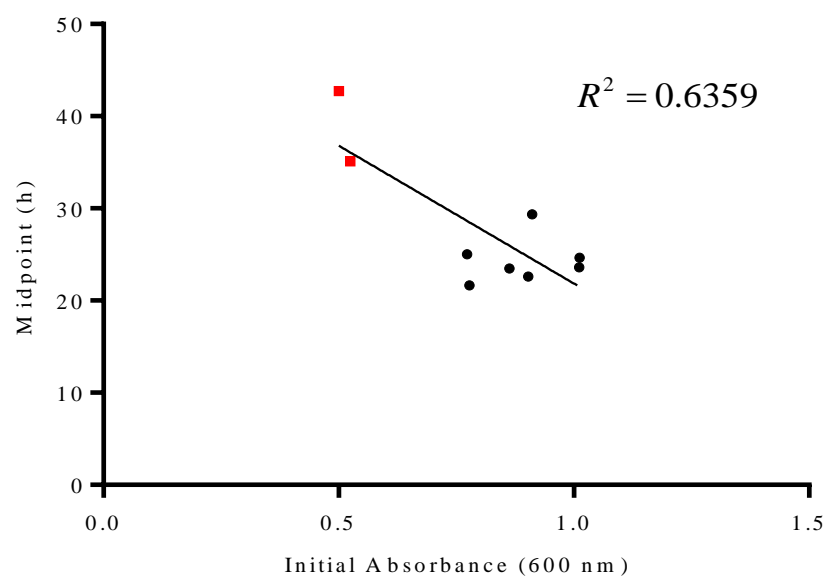
It was observed that fermentations four (a) and four (b) exhibited aberrant density attenuation rates compared to the others. The midpoint of fermentation (M) for 4a and 4b were higher than the other fermentations that suggested slower fermentations.



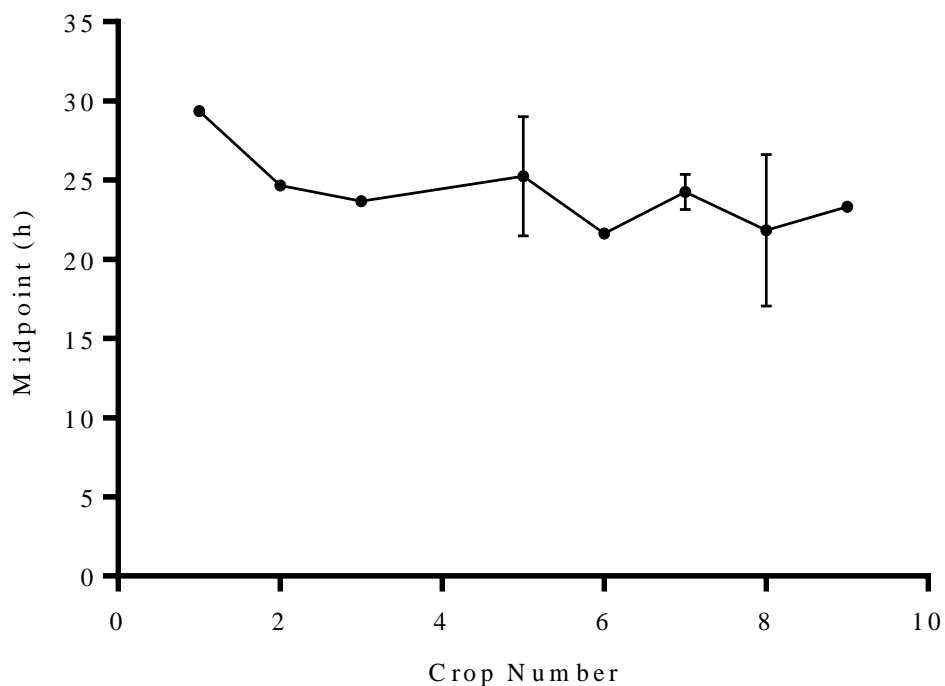
**Figure 4.2.** The density attenuation data for nine serial re-pitched fermentations was modelled using a four-parameter nonlinear regression. The density attenuation for fermentations two, three (a), three (b), and nine showed no significant ( $p > 0.01$ ) difference (green). Additionally, fermentations seven (a) and seven (b) showed no significant differences (orange) ( $p > 0.01$ ).

The two slowest fermentations, four (a) and four (b), were investigated. A significant ( $p < 0.05$ ) decreasing linear trend was found between the midpoint (h) of the fermentation and the absorbance (600nm) at time 0 (Figure 4.3).

To determine whether there was an overall change in fermentation rates as the yeast was serial re-pitched, the midpoint of the fermentation was compared to the crop number (Figure 4.4). Since the midpoint of fermentation four (a) and four (b) were influenced by the initial absorbance, they were removed from this analysis. It appeared that the midpoint of the fermentation decreased as the crop number increased (Figure 4.4), however an F-Test investigating whether the slope was non-zero found that the trend was not significant ( $p > 0.05$ ) and there were no linear correlations between the midpoint of the fermentation and the crop number of the yeast (Figure 4.4).



**Figure 4.3.** The time the fermentation was halfway complete (M) was predicted by the initial absorbance relating to the quantity of yeast at the start of fermentation. The midpoint (h) for the fermentation with crop 4 (red square) yeast was higher than all other yeast crops (black circles).



**Figure 4.4.** The time each re-pitched fermentation took to reach the midpoint (hour), determined by the four parameter nonlinear regression.

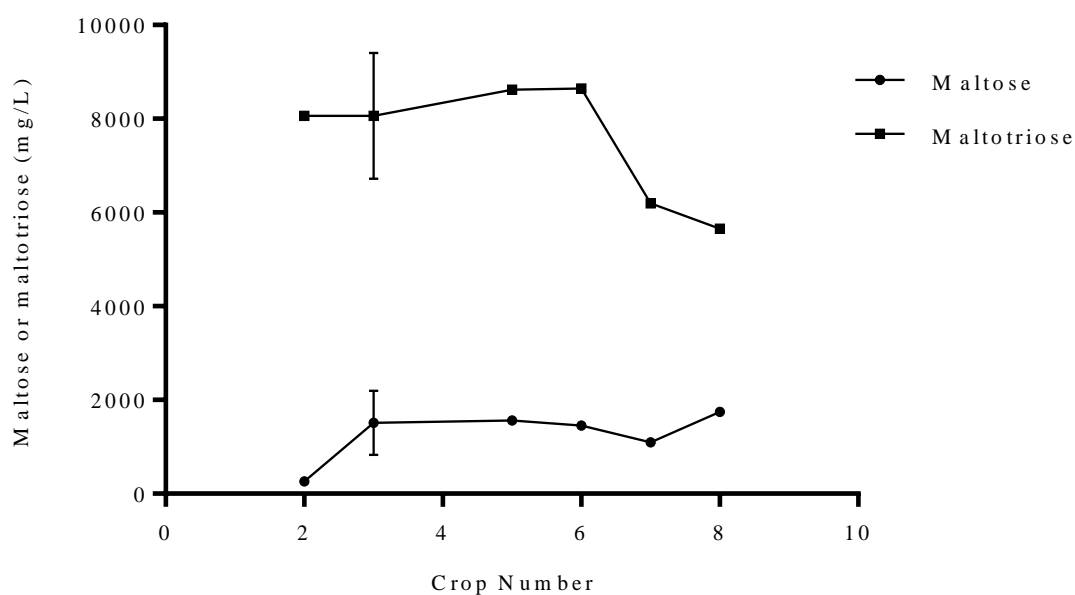
### 4.3.2 Carbohydrate Analysis

The concentrations of the principal brewing sugars were quantified at the start of fermentation (Table 4.2), maltose was present in the highest concentrations at  $30617 \pm 2444$  mg/L. Fructose was present in the lowest concentrations at  $830 \pm 104$  mg/L. When the fermentation was 95 % complete, the brewing sugars concentrations were also analysed from each re-pitched fermentation. The concentrations of maltose and maltotriose at 95 % complete fermentations was shown (Figure 4.5). There was a peak in maltose and maltotriose present when re-pitched fermentations were 95 % completed for crop 4, however these two fermentations were removed from analysis as explained earlier. No glucose, fructose, or sucrose remained in the fermenting beer when the fermentations were 95 % completed.

**Table 4.2.** Average concentration of individual carbohydrates (mg/L) at the start of fermentation.

Brew Number	Crop Number	Carbohydrates (mg/L)				
		Glucose	Fructose	Sucrose	Maltose	Maltotriose
66	2	4632	1001	1947	34915	13408
79	3	4475	970	1855	27942	11615
81	3	4673	825	1915	32961	12961
88	4	3825	833	1894	29995	12036
90	4	4103	796	1796	27937	11102
98	5	3875	805	1844	29303	11200
105	6	4398	835	1833	32053	14668
112	7	3920	667	1652	28647	12892
114	8	4010	735	1871	31804	12766
<b>Mean</b>		<b>4212</b>	<b>830</b>	<b>1845</b>	<b>30617</b>	<b>12516</b>
<b>SD</b>		<b>334</b>	<b>104</b>	<b>85</b>	<b>2444</b>	<b>1151</b>
<b>n</b>		<b>9</b>	<b>9</b>	<b>9</b>	<b>9</b>	<b>9</b>

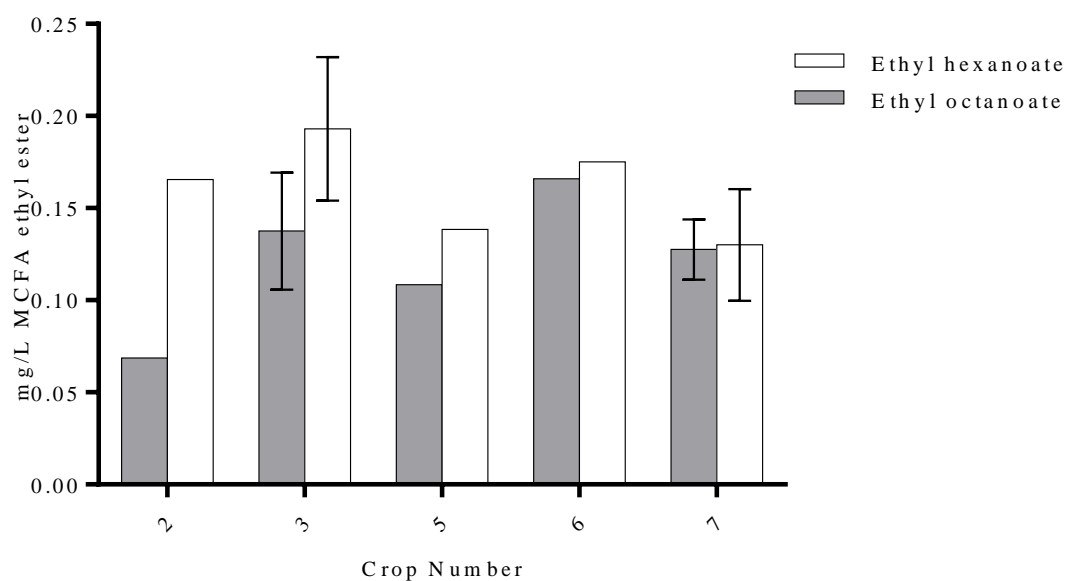




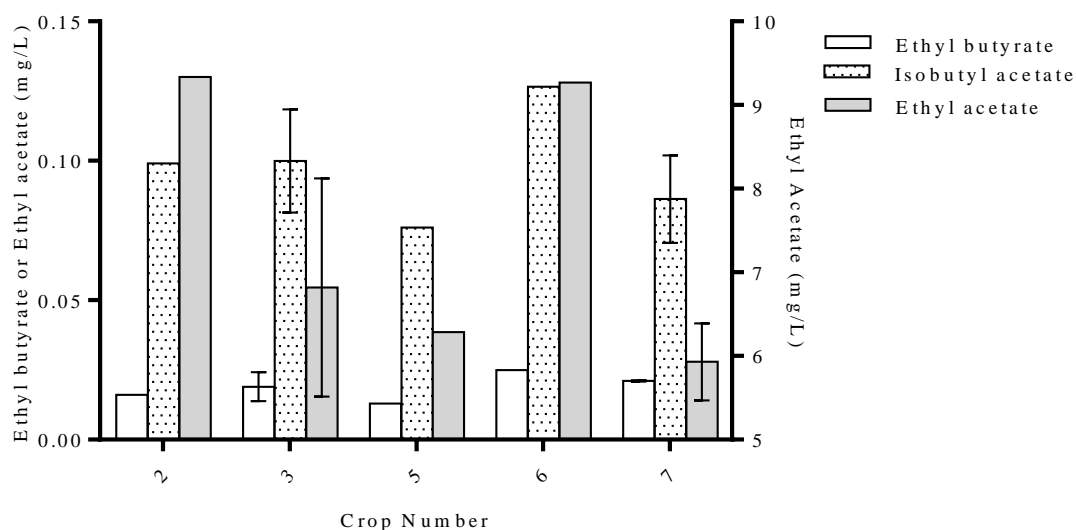
**Figure 4.5.** The concentration of maltose and maltotriose when the fermentation was 95 % complete for each of the re-pitched fermentation. There was no significant correlation between the crop number and maltose or maltotriose (mg/L).

### 4.3.3 Flavour Compound Analysis

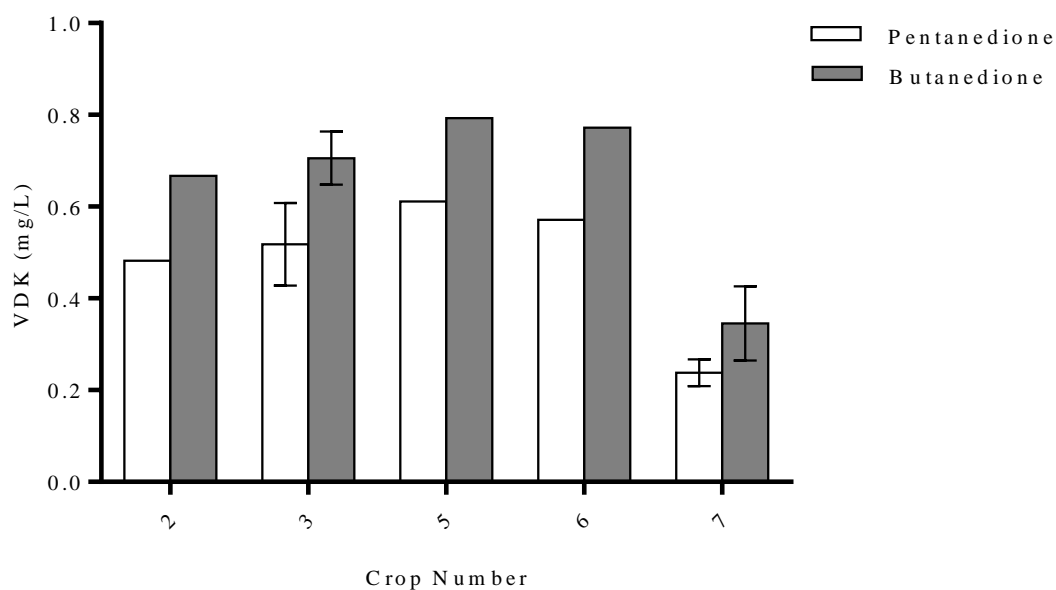
Vicinal diketones, acetate esters, ethyl esters, and higher alcohols were analysed when the fermentation was 95 % complete, up to fermentation seven. There were no observable trends between the crop number and the concentrations of ethyl hexanoate, and ethyl octanoate (Figure 4.6), and ethyl butyrate, isobutyl acetate, and ethyl acetate (Figure 4.7). The concentrations of 2,3-butanedione and 2,3-pentanedione appeared constant until the concentrations decreased for the fermentations with yeast crop seven (Figure 4.8), however the change was not found to be statistically different. The only flavour compound that showed a significant change as the crop number increased was propan-1-ol, exhibiting a linear decrease as the crop number increased (Figure 4.9).



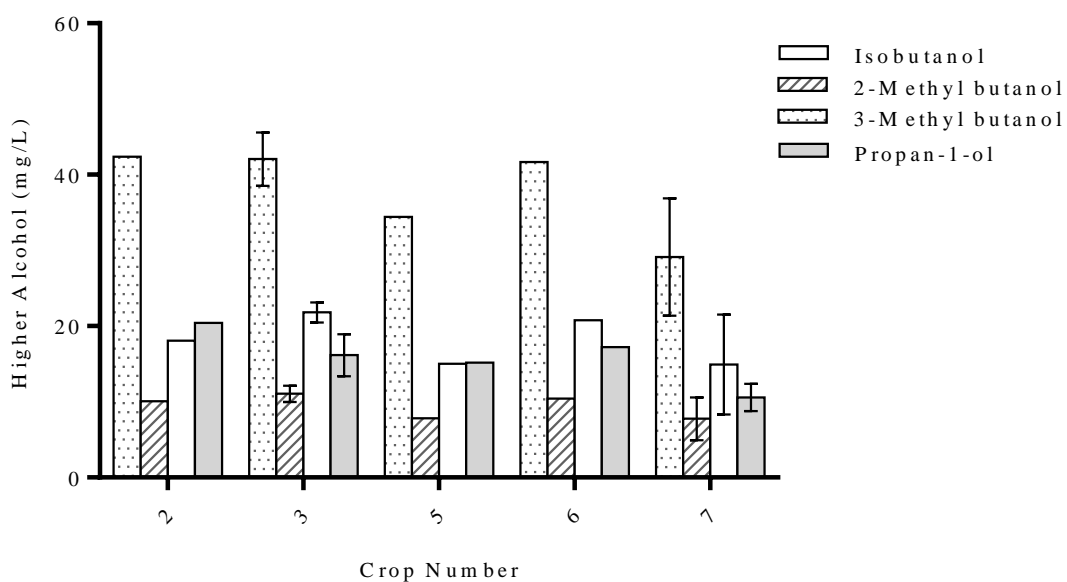
**Figure 4.6.** Levels of ethyl hexanoate and ethyl octanoate, present at 95 % completed fermentations as the yeast was serial re-pitched seven times. When duplicate fermentations were performed, the error bars represent the standard deviation.



**Figure 4.7.** Levels of acetate esters post 95 % completed fermentations after seven serial re-pitched ale fermentations. When duplicate fermentations were performed, the error bars represent the standard deviation.



**Figure 4.8.** Levels of vicinal diketones at 95 % completed fermentations as the yeast was serially re-pitched 7 times. When duplicate fermentations were performed, the error bars represent the standard deviation.



**Figure 4.9.** Levels of higher alcohols analysed at 95 % completed fermentations after seven serial re-pitched ale fermentations. When duplicate fermentations were performed, the error bars represent the standard deviation.

## 4.4 Discussion

### 4.4.1 Density Attenuation

In this study, brewery protocol was to discard the yeast culture after nine fermentations. In each of the fermentations monitored, most density attenuation modelled trends were significantly different.

The density attenuation rates for one, four (a), four (b), five (a), five (b), six, and eight were significantly different from the attenuation of all other fermentations. However, with the significantly different density attenuation rates between the stated fermentations, no observable trends indicated the differences were related to a continuously increasing crop number. There were likely numerous factors that contributed to this observation, the most likely of which are presented below.

The attenuation rate for the first fermentation was significantly different than the subsequent rates for the fermentations. It is known that freshly propagated yeast needs to adapt to fermentation conditions (Kobi *et al.*, 2004). The first fermentation utilized freshly propagated yeast that was grown in conditions with excess oxygen, excess nitrogen, and excess nutrients. Fermentation conditions differ from propagation, and consequently, the yeast culture adapts, responding to subsequent fermentations differently.

Fermentations four (a) and four (b) each, were significantly different from the other fermentations. It was observed that these two fermentations were the slowest. The pitching rate was uncontrolled during the brewing fermentation at this brewery, and therefore was monitored to determine how precise the pitching was between the fermentations. The pitching rate was not precise, and these slow fermentations correlated with two underpitched fermentations, when the cell count was checked.

More factors that likely contributed to the variation in density attenuation rates between re-pitched fermentations include initial individual sugar concentrations (Table 4.2), and initial dissolved oxygen level variation. In commercial brewing, these variations are common because ‘control’ is limited on a large scale. Each of these factors play a role in the density attenuation rates (D’Amore *et al.*, 1989; Verbelen *et al.*, 2009; Kucharczyk and Tuszyński, 2015). On top of these factors, this brewery’s specific practice of mixing yeast crops from multiple fermentations of different beers made it difficult to interpret the data. When the yeast was cropped from other fermentations for different brands of beer, these were combined into the same yeast storage tank prior to re-pitching. The multiple brands of beer produced from the same

yeast culture was likely another factor for significant attenuation rates found with certain crop numbers. In these cases, a portion of yeast culture was exposed to wort with differing compositions, nutrient availability and original gravities. A previous study demonstrated that slight differences in permutations of fermentation conditions, in this case varying oxygen levels, resulted in a change in yeast's gene expression (Bühligen *et al.*, 2013). It is likely that the varying conditions that portions of the re-pitched yeast culture experienced contributed to the significant changes observed in attenuation rates

There were many fermentations where the density attenuation rates were determined to be different. However, even with the plethora of factors that likely contributed to varying attenuation trends, some density attenuation curves were not significantly different ( $p > 0.1$ ). This was demonstrated for fermentation trends in yeast crops two, three (a), three (b), and nine. Additionally, the density attenuation curves between fermentation seven (a) and seven (b) were not statistically different. A previously published study investigated a series of industrial fermentations that were re-pitched 98 times (Powell and Diacetis, 2007), found no change in density attenuation trends during fermentation. In this case, the density attenuations rates did not change or drift with crop number, in fact the rates were not significantly different (Powell and Diacetis, 2007). These observations were confirmed by the results from the current study. This current study observed that fermentation nine had the same attenuation trends as earlier fermentations (two, three (a) and three (b)). The midpoint of the fermentation was also unchanged over the entire serial re-pitched cycle (Figure 4.4). This strengthened the argument no overall drift in fermentation attenuation rates with the serial re-pitching study was observed. Based on these observations, the density attenuation provided no insight towards a compromised fermentation due to the yeast crop number. This brewery stops at the 9<sup>th</sup> fermentation because of company protocol. It is hypothesized that this brewery could revise re-pitching protocols beyond the current limit of nine re-pitching's, which is not unheard of with ale serial re-pitching (Powell and Diacetis, 2007; Smart and Whisker, 1996).

#### **4.4.2 Carbohydrates**

Looking further into the sugar consumption of the yeast, the individual carbohydrates at the beginning and nearing the end of the fermentation were analysed. The uptake of sugars by yeast over fermentation occurs in a known order (Stewart and Russell, 2009; MacIntosh *et al.*, 2016). The approximate order that yeasts metabolize sugars during fermentation is sucrose, glucose, fructose, maltose and then maltotriose

(Stewart, 2016). The only two sugars that remained present in 95 % complete fermentations were maltose and maltotriose. No glucose, fructose or sucrose remained, which was as expected, because those sugars are metabolized first by the yeasts during fermentation (Phillips, 1955; MacIntosh *et al.*, 2016; Stewart, 2016). The concentrations of maltose and maltotriose that remained had no linear trends related the crop number. This result accompanies the density attenuation observations and reinforces that the sugar consumption of the ale yeasts was not affected by the crop number. This strengthened the initial hypothesis that the crop number from the nine serial re-pitched ale fermentations studied did not impact the green beer quality.

#### **4.4.3 Flavour Compounds**

In ale fermentations, high levels of higher alcohols, ethyl acetate and ethyl hexanoate are typical (Pires *et al.*, 2014). In the current study, both higher alcohols and esters were analysed post primary fermentation. Thorough analysis of the flavour compounds between two fermentations was difficult because only one sample was taken for flavour compound quantification per fermentation. This limited the options for statistical analysis.

Linear regression was performed to determine if there were any trends that related to the crop number. There were no significant differences in higher alcohols as the crop number increased, with the exception of propan-1-ol. Propan-1-ol levels post fermentation decreased as the crop number increased. The flavour threshold of propan-1-ol is 600 mg/L (Pires and Brányik, 2015). Even though the decrease in propan-1-ol was noted, the levels present were lower than the threshold, therefore this observation would likely be undetectable to consumers. A previously published study found an increase in 3-methyl butanol with serial re-pitching (Kobayashi *et al.*, 2007). This trend was not observed within this current study. The differing result was unsurprising because flavour compound production depends on several factors such as yeast strain and fermentation conditions. The published study used a lager strain of yeast, which was not subjected to acid washing. There are many differences in re-pitching with lager yeast and re-pitching with ale yeast (Table 4.1), other than the different yeast type.

Vicinal diketones are generally seen as off flavours. These compounds give beer a buttery or butterscotch flavour. In some beer styles, such a Bohemian pilsner and some English ales, vicinal diketones are desired (Krogerus and Gibson, 2013). The flavour threshold for 2,3-butanedione and 2,3-pentanedione and is 0.1 – 0.15 mg/L and 0.9 – 1.0 mg/L respectively (Pires and Brányik, 2015). One study analysed nine ales and

found 2,3-butanedione levels were between 0.05 mg/L – 0.3 mg/L and 2,3-pentanedione levels were between 0.01 mg/L – 0.2 mg/L (Harrison *et al.*, 1965). On average the 2,3-butanedione concentrations found in the current study post primary re-pitched fermentations were 0.61 mg/L  $\pm$  0.17. These concentrations would have decreased as the beer was conditioned and did not represent a true value of the concentrations of 2,3-butanedione in the final products. The crop number had no significant ( $p > 0.05$ ) impact on the levels of 2,3-butanedione of 2,3-pentanedione.

Most flavour compounds showed no significant linear changes in flavour compounds associated with the crop number. These results suggest that the flavour compounds produced with the serial re-pitched ale fermentations would not have affected the final beer quality. These results supported the hypothesis that the crop number did not impact the green beer quality.

## Chapter 5 – Small Scale Method Suitability for Serial Re-Pitching

### 5.1 Introduction

Large scale fermentations do not allow for the control and manipulation of variables that is possible on a small scale. Large scale fermentations are also high cost procedures. Small scale fermentations allow one to understand the impact of one variable, without the noise and variation of a large-scale fermentation. For this serial re-pitching study, a suitable small scale fermentation method was required. The method ASBC Yeast-14 allowed sufficient sampling during fermentation to collect data on the physical properties of the fermentation, such as the density attenuation and yeast in suspension. The EBC tall tubes fermentation method (European Brewery Convention, 2004) was also considered. This method however, was less attractive, because the volume needed to complete those fermentations was much larger than the ASBC Yeast-14 method. The larger volumes would have required multiple batches of wort to complete the re-pitching study and that could have introduced error into the study.

During brewery fermentations, density is the principal factor measured in industrial processes and the most widely-used method for measuring fermentation progression by breweries. Density can be measured by using a variety of equipment in the industry with a wide range of costs ranging from a simple hydrometer to a bench top densitometer with added features such as measurement of alcohol. The decrease in measured density is nearly proportional to the consumption of sugars by yeast and production of ethanol and CO<sub>2</sub> (Section 1.2.4) (MacIntosh *et al.*, 2016). Breweries aim to achieve final density attenuations between fermentations that are precise and consistent, between re-pitched fermentations. When the density attenuation rates differ from previous fermentations it may indicate quality problems in the final product (Kucharczyk and Tuszyński, 2015).

The fermentation must contain crucial nutrients needed for adequate yeast growth or the fermentations may become stuck (Bisson, 1999) and re-pitching would not be a viable proposition. For energy, yeasts utilize carbon sources, which come from the brewing wort sugars: maltose, glucose, sucrose, fructose, and maltotriose (Section 1.3.1). Nitrogen is necessary for yeast growth in form of amino acids, peptides, amines, pyrimidines, purines (Section 1.3.4) (Briggs *et al.*, 2004). In brewing fermentations, most of the nitrogen sources come from FAN. When levels of FAN in the wort are sub



optimal, the fermentation may exhibit slow and/or incomplete attenuation rates (Krogerus and Gibson, 2013). Jones and Rainbow (1966) suggested that normal wort gravities need 140 – 150 mg/L FAN, and in high gravity worts, higher FAN levels are necessary (O'Connor-Cox and Ingledew, 1989). In much smaller quantities yeast also need metals such as magnesium, calcium, and zinc (Aleksander *et al.*, 2009; De Nicola and Walker, 2009). Other metals which yeast utilizes during fermentation are boron, calcium, cobalt, copper, iron, potassium, manganese, magnesium, nickel (Briggs *et al.*, 2004). Generally, brewer's malt wort contains all minerals necessary (Briggs *et al.*, 2004), with the exception of zinc (Daveloose, 1987).

## 5.2 Experimental Design

Two series of re-pitching experiments were completed using an adapted version of ASBC Yeast-14 (Section 2.3). The first re-pitching series utilized frozen prepared wort from batch 1501 (Section 2.2.1). The fermentations were set up, monitored, and modelled according to the adapted version of ASBC Yeast-14 (Section 2.3) using a final volume of 944 mL of wort per batch. This resulted in a total of 47 test tube fermenters, of which 30 tubes were used for destructive sampling to determine the fermentation characteristics described in an earlier chapter (Section 2.3.2). The final 17 tubes were utilised at the end of fermentation to collect yeast for re-pitching. From these 17 test tubes, the beer was removed using a 5 mL pipette leaving the yeast sediment at the bottom. A 40 mL portion of removed beer was saved in a 50-mL sterile centrifuge tube. Next, a 5 mL pipette was utilized to collect the top portion of yeast sediment that remained in the test tubes. Extreme caution was taken not to collect the yeast on the bottom layer of the test tube. This method mimicked the yeast cropping methods used for industrial brewing fermentations. The cropped yeast from the 17 test tubes were combined into one 50 mL centrifuge tube. The saved 40 mL aliquot of beer was transferred into the centrifuge tube containing the yeast to provide storage media. The lid was replaced on the tube, sealed with Parafilm, and stored at 4 °C for 41 hours before re-pitching. Prior to re-pitching, the beer on top of the stored yeast was decanted from the centrifuge tube. Approximately 30 mL of distilled water was added to the remaining yeast pellet and mixed. The tube was centrifuged at 3 000 x g for 3 min. The supernatant was removed and the cells were re-suspended in approximately 30 mL of distilled water and mixed. This process was repeated until the cells were “washed” a total of three times as described earlier (Section 2.3.1). The cells were enumerated and viability tested using a haemocytometer and methylene blue dye for re-pitching (Section

2.5.2). The series of fermentations were completed in duplicate with two different lager strains, SMA and W34/70. Fermentations with strain W34/70 were re-pitched three times and fermentations with strain SMA were re-pitched four times. When SMA was re-pitched for the fourth time, on this occasion, the duplicate fermentations were given different treatments of oxygen. For one treatment, the wort was oxygenated by bubbling oxygen at approximately three psi through stirred wort for five minutes, and the second treatment was given 20 minutes of bubbling oxygen at approximately 3 psi. In addition to the fermentation characteristics monitored as previously described, the FAN levels, and zinc levels in the wort were determined. The levels of zinc in the brewery water, boiled wort, and boiled and autoclaved wort were determined (Section 2.6). The FAN levels were determined by the ninhydrin method (Section 2.7).

The second series of re-pitched fermentations utilized another wort batch, 1521 (Section 2.2.2). In the case of this wort, the mashing regime was adjusted to achieve higher levels of FAN in the wort of 276.8 mg/L of FAN, as opposed to 181.9 mg/L FAN in the previous batch. The fermentations were set up and analysed according to ASBC Yeast-14 (Section 2.3) with a final volume of 1000 mL wort per batch. The yeast collection method was adjusted from the first experiment. Collecting the yeast from multiple small test tubes created an increased risk for contamination. As well, the size of the sediment at the bottom of the tubes was difficult to collect the yeast in a manner that mimicked the yeast collect methods in a brewery where the bottom layer is not collected, and the middle to upper portion is. The solution devised was to perform two fermentations in larger fermenters. These vessels were glass cylinders with a larger diameter and conical bottom. Previously published literature had demonstrated that the height of the fermenter is what drives the speed of fermentation due to the evolution of CO<sub>2</sub> (Lake *et al.*, 2008), therefore the height of the wort in these experiments was designed to remain the same for both fermenters regardless of the differences in diameter of the fermenters (MacIntosh *et al.*, 2012).

Yeast collection for this second series of re-pitching experiments replicated the conditions of the first, but with a larger volume. The beer was removed by a 20 mL sterile pipette and a portion was saved for a yeast storage medium. The top and middle section of the yeast sediment was collected by using a 5 mL pipette and placed into a 50 mL sterile centrifuge tube. The beer saved from the fermenter was used as the medium to store the yeast. The yeast storage tube was sealed with parafilm and placed at 4 °C for 41 hours before re-pitching. Before re-pitching into the next fermentation, the yeast

was washed and counted (Section 2.5.2). This series of fermentations was re-pitched seven times. At the end of these fermentations, the cells were analysed to determine the average cell age through fluorescence microscopy to enumerate the bud scars (Section 2.8). The second series of re-pitched fermentations was completed once for each yeast strain, SMA and W34/70.

The experimental work presented here determined the consistency of density attenuation rates between re-pitched fermentations using modelling. Also, the suitability of Yeast-14 to determine the fermentability of yeast in re-pitching studies was investigated.

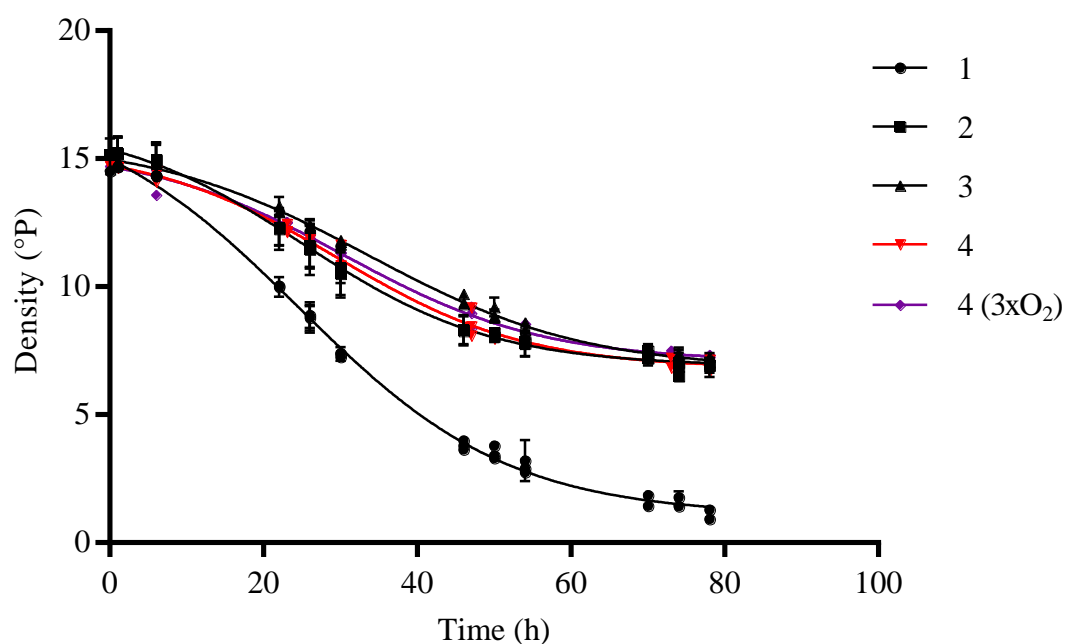
### **5.3 Results**

#### **5.3.1 *Fermentation Series One: Four Re-Pitches***

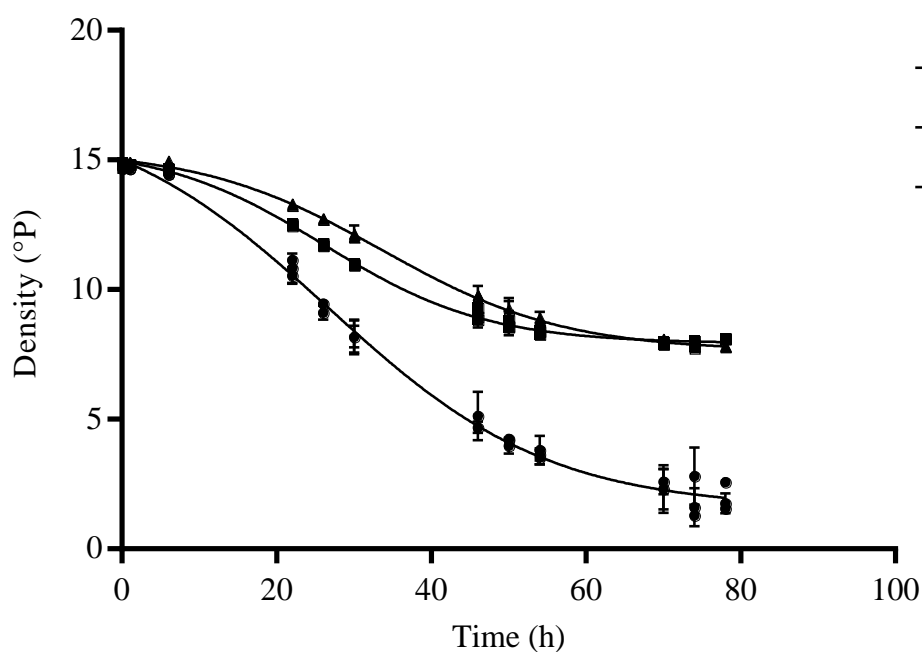
All re-pitched fermentations in the first series of fermentations were sluggish, and could be described as ‘stuck’ for both yeast strains SMA (Figure 5.1) and W34/70 (Figure 5.2). Stuck fermentations do not reach the desired final density attenuation. The initial fermentations for strain SMA with freshly propagated yeast reached a final density of approximately 1 °P, whereas the re-pitched fermentations reached a final density of approximately 6 °P at the end point of the fermentation experiment. The stuck fermentations with strain W34/70 were more pronounced. The re-pitched fermentations reached a final density of approximately 8 °P and by comparison, the freshly propagated yeast reached a final density of approximately 1 °P.

In addition to the density attenuation results, neither strains during the re-pitching cycle reached the same maximum absorbance as when using the freshly propagated yeast (Figure 5.3 and 5.4). The maximum peaks for the absorbance with strain SMA for re-pitched fermentations were between 1.43 and 1.72 and the maximum absorbance peak when using propagated yeast was 2.12. With yeast strain W34/70, the maximum absorbance peaks for re-pitched fermentations were between 1.64 and 1.66 however the maximum absorbance for the fermentation with propagated yeast was 2.04.

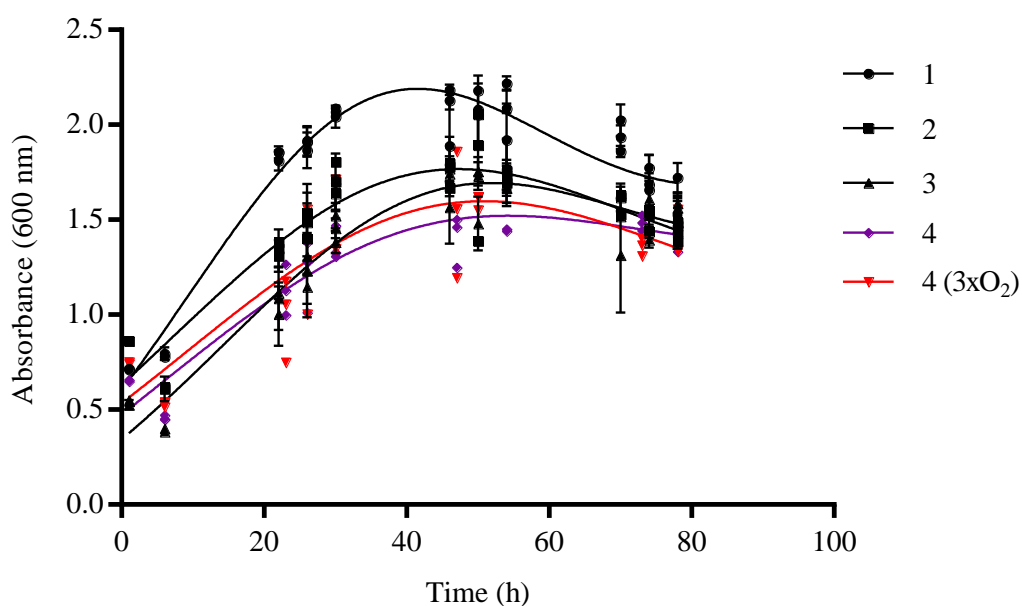
Finally, the change in absorbance that could be attributed to yeast growth within the fermentations was calculated (Table 5.1). This calculation was the difference between the maximum absorbance and the absorbance at time 0 of the fermentation. The fermentations that had the greatest amount of yeast growth were the fermentations carried out with freshly propagated yeast (SMA and W34/70), which had a difference in absorbance of 1.404 and 1.370 respectively.



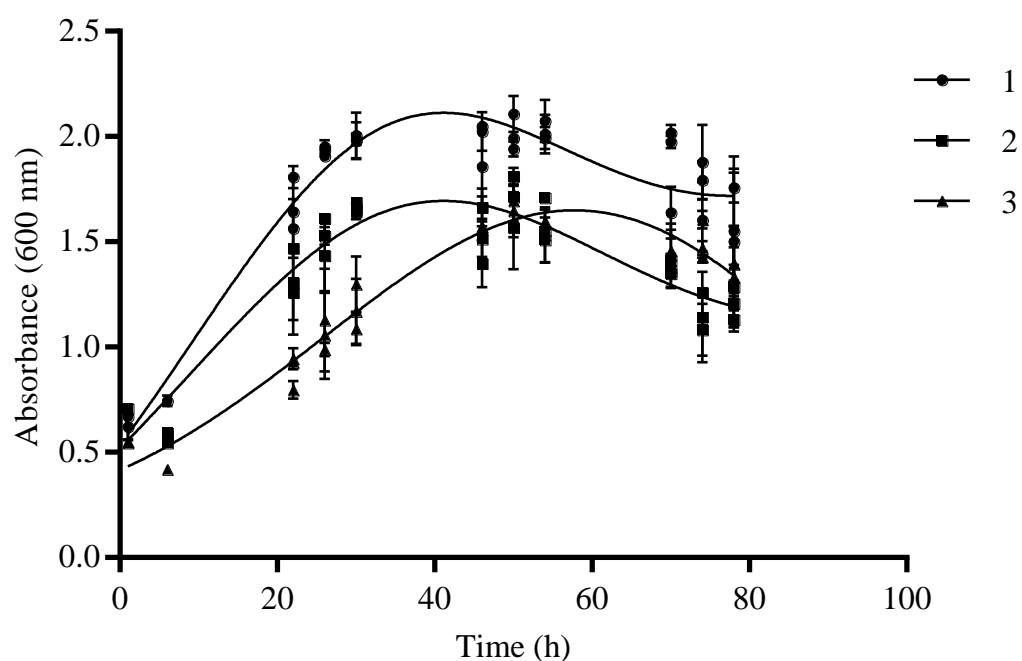
**Figure 5.1.** Modelled density attenuation trends for four serial re-pitched fermentations with strain SMA completed in duplicate. The last re-pitched fermentation (4) was given two oxygenation treatments instead of the duplicates, one had the same oxygenation levels as the previous fermentations and the other was given 3 x O<sub>2</sub> as the previous fermentations (3 x O<sub>2</sub>). The error bars represent the standard deviation of the duplicate fermentations.



**Figure 5.2.** Modelled density attenuation trends for three serial re-pitched fermentations with strain W34/70 completed in duplicate. The error bars represent the standard deviation of the duplicate fermentations.



**Figure 5.3.** Modelled absorbance (600nm) trends for four serial re-pitched fermentations in with strain SMA. The last re-pitched fermentation (4) was given two different oxygenation treatments. One treatment had the same oxygenation levels as the previous fermentations. The other treatment was given 3 x O<sub>2</sub> as the previous fermentations (3 x O<sub>2</sub>). The error bars represent the standard deviation of the duplicate fermentations.



**Figure 5.4.** Modelled absorbance (600nm) trends for three serial re-pitched fermentations with strain W34/70. The error bars represent the standard deviation of the duplicate fermentations.

**Table 5.1.** The maximum absorbance and the absorbance at time 0 in each fermentation were determined. The difference in these two measurements was calculated to determine the absorbance that was attributed to yeast growth.

Yeast Strain	Fermentation	Time 0 Absorbance <sup>a</sup>	Maximum Absorbance <sup>b</sup>	Δ Maximum Absorbance and Time 0 Absorbance
SMA	1	0.716	2.12	1.404
SMA	2	0.862	1.72	0.858
SMA	3	0.544	1.63	1.086
SMA	4 (15 x O <sub>2</sub> ) <sup>c</sup>	0.649	1.43	0.781
SMA	4 (5 x O <sub>2</sub> ) <sup>c</sup>	0.751	1.56	0.809
W34/70	1	0.670	2.04	1.37
W34/70	2	0.707	1.66	0.953
W34/70	3	0.544	1.64	1.096

<sup>a</sup>The absorbance at time 0 was a better predictor of the pitched yeast than the absorbance at time 0 calculated from the model. For that reason, the data at time 0 was used instead of the model

<sup>b</sup>The model was used to calculate the maximum absorbance at a time =  $\mu$  (Section 2.3.3). This was the time when the peak was the highest that calculated the maximum absorbance.

<sup>c</sup>Initially oxygenated for 5 or 15 minutes (see text).

The zinc in mains water varied between 0.053 to 0.68 mg/L depending on the time sampled. The levels of zinc in boiled wort were higher than the tap water at  $0.94 \pm 0.04$  mg/L. Boiled and autoclaved wort for wort batch 1501 had the highest levels, at  $1.11 \pm 0.16$  mg/L (Table 5.2.). The FAN levels in autoclaved wort for batch 1501, and diluted to 90 %, were  $181.9 \pm 0.1$  mg/L (Table 5.3). When the wort was boiled and not autoclaved, and diluted to 90 %, the levels of FAN were between  $204.1 \pm 17.0$  mg/L (Table 5.3). Prior to fermentation, water was added to adjust the total volume of the fermentations and would result in the dilution of FAN levels. Therefore, the final FAN levels were calculated to include this dilution (Table 5.3).

**Table 5.2.** Zinc concentration in autoclaved and boiled wort (AC), only boiled wort (B), and tap water (T) for batch 1501

Sample	Mean	SD	RSD (%)	Rep			Mean (w/dil.)	Mean of Replicates (mg/L)
				1	2	3		
AC1	0.073	0.002	2.8	0.07	0.073	0.074	1.2775	1.108333
AC2	0.055	0.0061	11	0.062	0.052	0.051	0.9625	
AC3	0.062	0.0028	4.6	0.06	0.065	0.062	1.085	
B1	0.053	0.0036	6.8	0.05	0.051	0.057	0.9275	0.939167
B2	0.056	0.0018	3.2	0.056	0.054	0.058	0.98	
B3	0.052	0.0019	3.6	0.05	0.052	0.053	0.91	
T1	0.068	0.0012	1.8	0.069	0.067	0.067	0.068	0.053
T2	0.01	0.0063	6.3	0.104	0.103	0.092	0.01	
T3	0.081	0.0031	3.8	0.079	0.08	0.085	0.081	
T4	0.689	0.0048	0.7	0.683	0.693	0.69	0.689	0.6765
T5	0.664	0.0065	1	0.657	0.667	0.669	0.664	

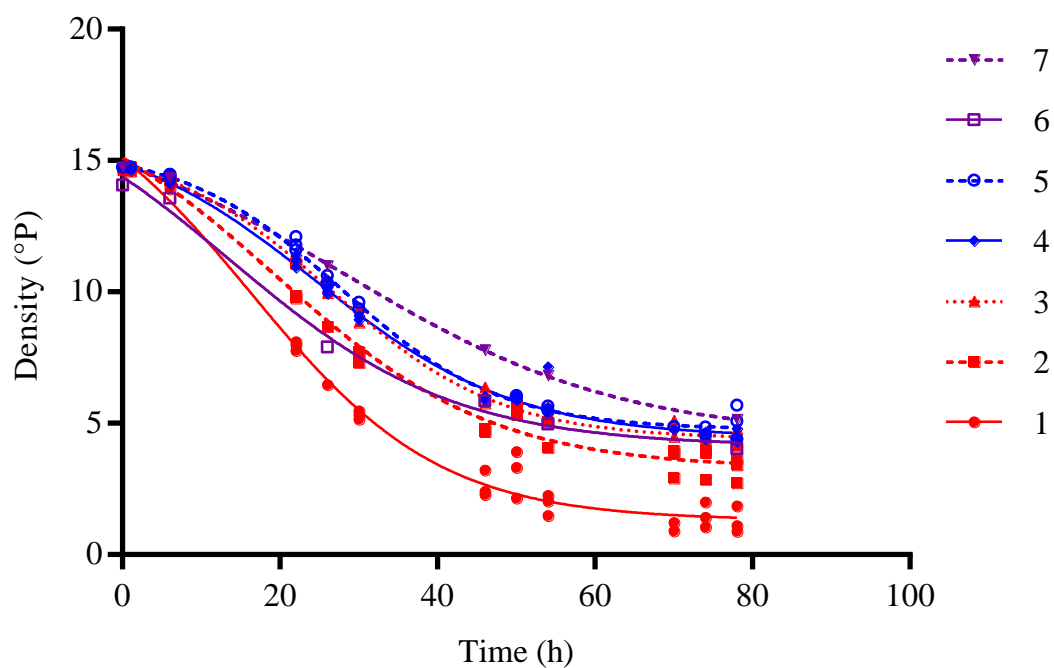
**Table 5.3.** FAN levels in worts that were either boiled for one hour (boiled wort) or boiled for one hour and autoclaved (autoclaved wort) with batch 1501.

Sample	Average	SD	Net	FAN mg/L	After diluting to 90 %
DW <sup>a</sup>	0.048	0.013			
Glycine	0.5267	0.008	0.48		
Boiled Wort	0.5587	0.019	0.51	213.37	192.03
	0.6227	0.164	0.57	240.11	216.10
Autoclaved Wort	0.5317	0.002	0.48	202.09	181.88
	0.532	0.006	0.48	202.23	182.01

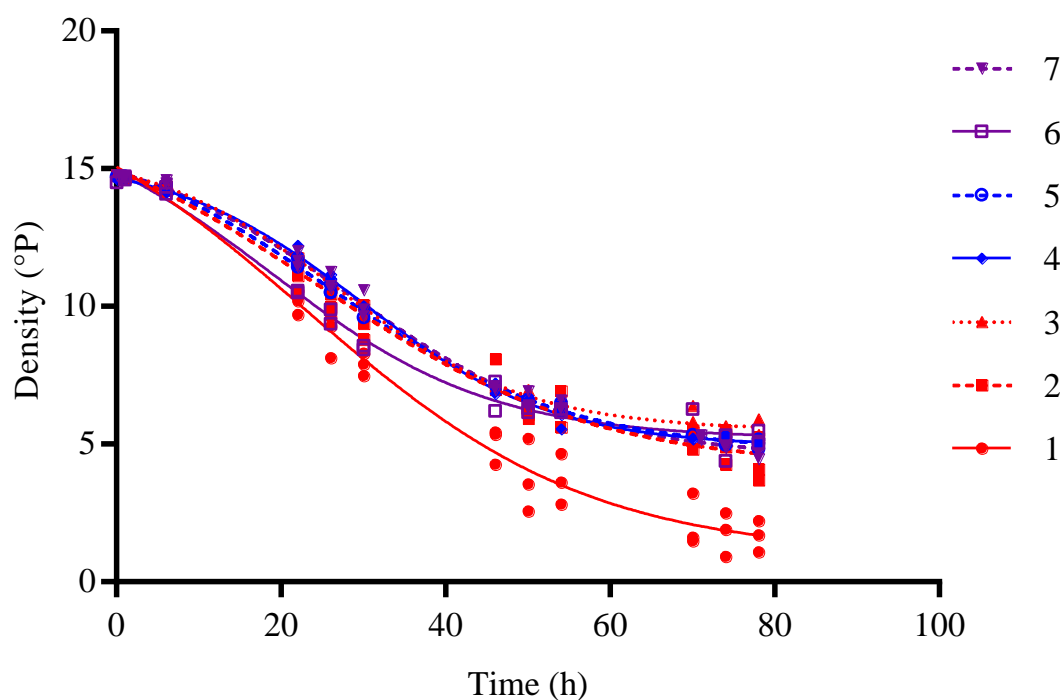
<sup>a</sup>DW = Distilled water

### 5.3.2 Fermentation Series Two: Seven Re-Pitches

The density attenuation rates were monitored throughout seven consecutive re-pitched fermentations for two strains of lager yeast, SMA (Figure 5.5) and W34/70 (Figure 5.6). Trends showed that the first fermentation for both yeast strains, SMA and W34/70, resulted in a lower final gravity of 1 °P, when compared to its re-pitched fermentation profile. The subsequent fermentations did not reach the same final gravity and arrested at approximately 5 °P.



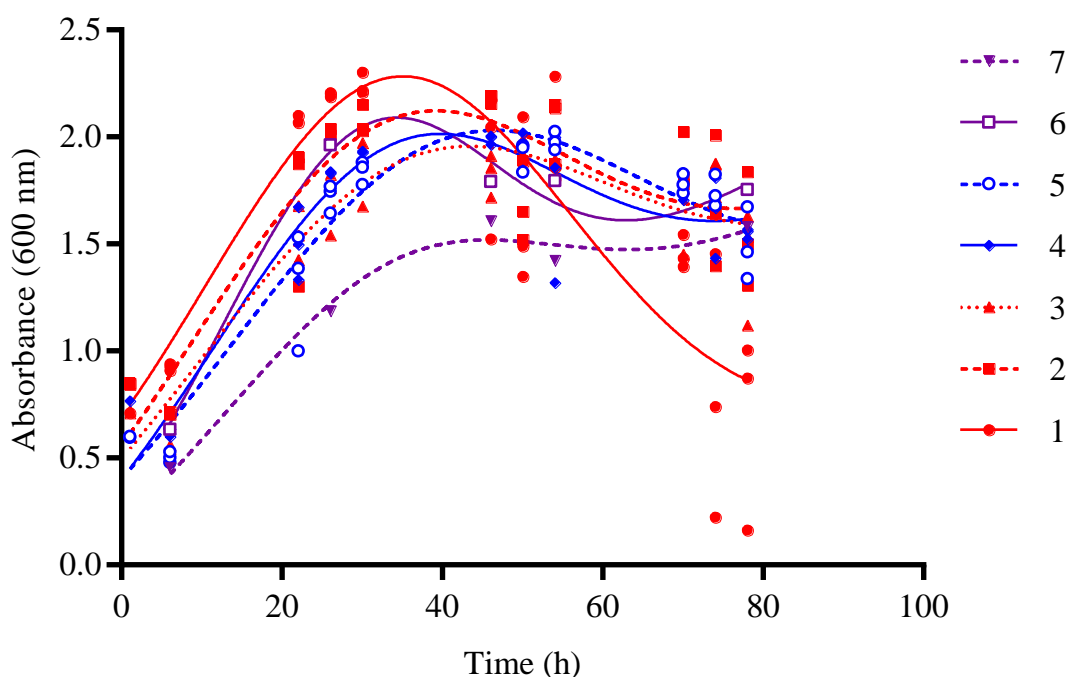
**Figure 5.5.** Modelled density attenuation trends for seven serial re-pitched fermentations using strain SMA. The wort contained higher levels of FAN than the previous series of re-pitched fermentations.



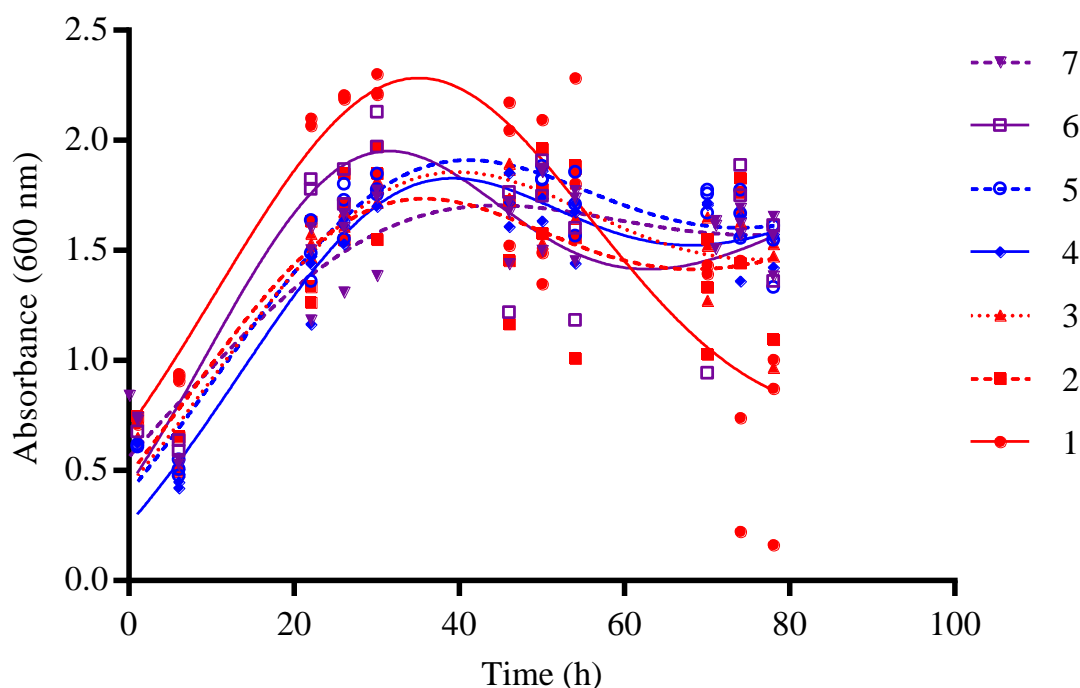
**Figure 5.6.** Modelled density attenuation trends for seven serial re-pitched fermentations using strain W34/70. The wort contained higher levels of FAN than the previous series of re-pitched fermentations.



In addition to the density, the absorbance trends (600nm) were monitored throughout the fermentations for each yeast strain (Figure 5.7 and Figure 5.8). The absorbance was a measurement of yeast in suspension, from hour 0 to approximately 40 of the fermentation. Once the absorbance peaked between 1.417 to 2.276, depending on the fermentation, the absorbance decreased for the remainder of the fermentation to 0.680 – 1.755. Fermentation one for both yeast strains peaked with the largest absorbance (2.276) and dropped to the lowest absorbance by hour 78 (0.680).



**Figure 5.7.** Modelled absorbance (600nm) trends representing the yeast in suspension over the course of the miniature fermentation with strain SMA for each serial re-pitched fermentation as designated by colour.



**Figure 5.8.** Modelled absorbance (600 nm) trends representing the yeast in suspension over the course of miniature fermentations with W34/70 for each serial re-pitched fermentation as designated by colour.

Finally, the FAN levels in batch 1521 were 276.8 mg/L and higher than the FAN levels wort batch 1501 at 182.0 mg/L when diluted for fermentation (Table 5.5).

**Table 5.5.** FAN in wort with protein rest in mash for fermentation in wort batch 1521.

Sample	Average	SD	Net	FAN mg/L	After diluting to 90 %
DW	0.0617	0.005			
Glycine	0.5023	0.007	0.4407		
Boiled Wort	0.7487	0.019	0.687	311.80	280.62
Autoclaved Wort	0.7393	0.02	0.6777	307.56	276.80

## 5.4 Discussion

### 5.4.1 Fermentation Series One

The re-pitched fermentations in series one fermentations exhibited stuck fermentation profiles. Fermentation one, which contained freshly propagated yeast, reached a lower final density than of the subsequent re-pitched fermentations two to four. The definition of a stuck fermentation is: fermentable sugars remain at the end of fermentation and leave higher levels of residual sugars than desired (Bisson, 1999). The

re-pitched fermentations in the current study stopped at approximately 7 °P for fermentations two and three. When these values were observed, the density had reached an equilibrium.

During the beginning of fermentation from hours 0 to approximately 40, the number of yeast cells in suspension increase as the cells grow and divide in the fermentation (White and Munns, 1951). Healthy fermentations exhibit at least a two to three fold cell growth during the first half of the fermentation in suspension of the fermenting beer (Boulton and Quain, 2001). An increase in absorbance was observed during the first half of fermentation, which related to yeast growth. In fermentation one from the first series of re-pitched fermentations, the yeast growth was the largest; the maximum absorbance was 2.12 and 2.04 for strain SMA and W34/70 respectively. The re-pitched fermentations reached a maximum absorbance between 1.43 – 1.72 with strain SMA and 1.64 – 1.66 with strain W 34/70. These results showed that yeast growth was reduced in the re-pitched fermentations compared to the fermentations with freshly propagated yeast. The reduction in yeast growth was accompanied with stuck fermentations and therefore, it was hypothesized that an essential nutrient may have been deficient in the brewing wort.

The level of wort oxygenation was investigated on the basis that dissolved oxygen plays a role in the amount of yeast growth (Kirsop, 1974). The duplicate fermentations for fermentation four in the series with yeast strain SMA were given two different oxygen treatments. One batch was given the same treatment of oxygen as the previous fermentations. The second batch was treated with three times the amount of normal oxygen by bubbling oxygen at three psi, through the stirred wort for 15 minutes. It was expected that if the oxygenation was insufficient, then a quicker density attenuation rate and more yeast growth would be observed in the fermentation with more oxygen. This was not observed. In fact, the maximum absorbance for fermentation four with three times the oxygen was 1.434 and the maximum absorbance for the fermentation with the normal oxygen treatment was 1.564. Therefore it was assumed the original oxygenation treatment had reached dissolved O<sub>2</sub> saturation. Sometimes, depending on the yeast strain, too much oxygen can be detrimental to yeast growth (Jakobsen and Thorne, 1980). This was unlikely the case for these strains because the first fermentation went to completion. If too much oxygen was present in the wort that impacted the yeast, the first fermentation would have likely been impacted as well.

Stuck fermentations may also occur when zinc is deficient in the wort (Helin and Slaughter, 1977). Zinc plays an important role in fermentation rates. If levels are too low, protein synthesis may become compromised since zinc is a crucial factor in enzymes involved in that pathway (De Nicola and Walker, 2009). Additionally, the phospholipid composition of the membranes may become impaired (De Nicola and Walker, 2009). Upon analysis of zinc levels in the wort in this study, it was shown that the zinc levels were likely not the limiting factor for these fermentations. The concentration of zinc in the autoclaved wort was 1.11 mg/L. Generally, stuck fermentations caused by insufficient zinc have concentrations below 0.1 mg/L (Helin and Slaughter, 1977; De Nicola and Walker, 2011). Zinc concentrations in the wort were over 10 times higher than levels that attribute to stuck fermentations and therefore likely not a limiting factor in these fermentations.

An important macronutrient for yeast growth in the fermentation is FAN (Stewart *et al.*, 2013). As the yeast growth declined during the re-pitched fermentations, it was hypothesized that FAN levels might have been insufficient for yeast growth. The ninhydrin method showed that the nitrogen levels for batch 1501 were 181.95 mg/L in the boiled and autoclaved wort. The boiled and autoclaved wort was analysed for FAN levels because heating the wort creates hot break that precipitates proteins (Briggs *et al.*, 2004). The FAN content was 181.95 mg/L and this level was above the recommended 140 – 150 mg/L for normal density worts of original density < 16 °P (Gibson, 2011). However, this wort started with an original density of approximately 16.1 °P, and therefore might require higher levels of FAN than a normal density wort. If the FAN was insufficient for these experiments, the high yeast growth in fermentation one (Figure 5.3 and 5.4) could be explained by the initial pitched yeast containing enough FAN, stored intracellularly, from yeast propagation. It was hypothesized that the FAN levels were insufficient in the wort. FAN is required for healthy yeast growth during fermentation and low levels can be associated with slower fermentations (Lekkas *et al.*, 2005). With the small-scale fermenters, if the fermentations are not quick enough then yeast settling may be a problem because the shear rate of the fermenter is lower than that of a large-scale (MacIntosh *et al.*, 2012). It was hypothesized that the slow fermentations may be related to a combination of both the low FAN levels and the size of the fermenter. Therefore, for the second series of fermentations, the mashing regime was tailored so the wort would contain a higher FAN level.

Along with the stuck fermentations, another issue faced was acquiring sufficient yeast to pitch into the succeeding fermentations. Given that 660 mL of the 944 mL wort was distributed into test tubes for destructive sampling, the remaining 284 mL of wort needed to provide enough yeast to pitch the subsequent 944 mL fermentation. In a typical fermentation, normally the cells divide two to three times (Powell *et al.*, 2008). With the reduced yeast growth observed in the re-pitched fermentations, the yeast volume collected for re-pitching was inadequate. The experimental design relied on healthy yeast growth during fermentation to collect for the subsequent fermentation. For the second fermentation series, the volume of wort utilized for fermentation was increased to provide the potential for more yeast to collect for re-pitching.

#### **5.4.2 Fermentations Series Two**

For the second series of re-pitched fermentations, two changes were made (1) higher FAN levels in the wort, and (2) a larger fermentation volume to provide more yeast for re-pitching.

It was hypothesized that with higher FAN levels, cellular growth in the fermentations would be consistent between re-pitched fermentations and re-pitched fermentations would not become stuck. The final density levels were lower than the values observed from the first fermentation series; however, they were still considered stuck. The lower asymptotic value,  $P_e$ , for fermentation one was 1.31 °P. The parameter  $P_e$  for fermentation two was 3.3 °P, and the subsequent re-pitched fermentations were 4.12 – 4.79 °P. This was an improvement compared to the stuck fermentations in the first fermentation series that reached final densities of approximately 7 °P. It was hypothesized that this is unique to the small scale re-pitched fermentations. The was suggested to be related to the height of the fermentations being too small for the re-pitched fermentations. Each fermenter was 20 mL with a height of 9 cm and it was previously demonstrated (MacIntosh *et al.*, 2012) that the miniature fermentation assay (ASBC Yeast-14) was good representation of industrial fermentations, with the exception of shear stress. MacIntosh and colleagues (2012) mentioned that in the test tube fermentations once yeast had settled, it was not easily re-suspended. In the current study, the yeast in suspension in the re-pitched fermentations declined between the hour 0 and hour 6 measurement (Figure 5.3, 5.4, 5.7, and 5.8). This was not observed with fermentation one and the freshly propagated yeast. It was hypothesized that yeast settled at the start of the re-pitched fermentations and was unable to become re-suspended through the natural agitation of CO<sub>2</sub> evolution. It is hypothesized that this effect

lowered the pitching rate and created slower fermentations. Considering this effect and the difficulties of collecting an adequate mass of yeast for re-pitching, this method and approach was considered unsuitable for the future re-pitching studies in this thesis.

These experiments demonstrated that the miniature fermentation assay (ASBC Yeast-14) was impractical for this study. However, one noteworthy observation was made. The re-pitched density attenuation trends for strain W34/70 were more precise than strain SMA. Strains all exhibit different characteristics that contribute to the uniqueness of the beer. Some of these characteristics are an advantage to the yeast during fermentation because they may have a higher ability to flocculate, which aids with crop collection at the end of fermentation. Some strains may cope with stresses better than others (Gibson *et al.*, 2007), which may make one strain more suitable for re-pitching than another. The re-pitched fermentations for W34/70 were more consistent than SMA, which was observed (Figure 5.5 and 5.6). This difference showed that there was less variation between the re-pitched fermentations for yeast strain W34/70 and the fermentative ability of this strain was more precise than yeast strain SMA.

## Chapter 6 – The Effect of Low Concentrations of Petite Mutations in Brewing Fermentations

### 6.1 Introduction

The development of respiratory deficient (RD) yeasts, also termed petites, occurs at different points during the brewing process (Jenkins *et al.*, 2009; Lawrence *et al.*, 2012, 2013). Higher frequencies of petites were found in the yeast sediment of the fermenter cone at the same location as flocculated older cells (Lawrence *et al.*, 2013). This same study found that older cells were more susceptible to artificial petite mutation induction with ethidium bromide than virgin cells (Lawrence *et al.*, 2013). Petites can also form during cell storage for re-pitching. A study observed that increased storage times led to increased frequencies of petites (Jenkins *et al.*, 2009). This study showed that day three to four was the critical time when petite mutation frequencies were increased (Jenkins *et al.*, 2009). One report showed that yeast cells stored for two weeks had a petite mutation frequency in the culture that reached 50 % (Morrison and Suggett, 1983). It is unclear whether this yeast had been warm cropped or exposed to high ethanol and high temperatures in the cone.

There are two genotypes of petite mutations, *rho*- that lack large sections of mitochondrial DNA (mtDNA) or *rho0* that lack the entire mtDNA (Merico *et al.*, 2007). Petites with the genotype *rho0* have been discussed in literature, and it was only recently that the *rho0* mutation has been found naturally in brewery fermentations (Lawrence *et al.*, 2012). Research with flor yeasts has shown that the *rho*- mutation can change into complete loss of mtDNA over time (Castrejón *et al.*, 2002). The mutations are spontaneous, however, external factors may stress the cells and as a result may encourage the formation of the mutations. Where each *rho*- mutation is unique, the impact that this mutation has on fermentation may be unique to the specific fermentation.

High frequencies of petite mutants, however, do not naturally exist in brewing yeast. One brewery reported that petite mutation frequency could range from 1 to 4 %, while another brewery encountered lower rates of 0.1 to 0.6 % (Morrison and Suggett, 1983). There has been a report of higher levels when cultures of yeast were re-pitched. Jenkins and colleagues observed that when lager yeast, *Saccharomyces cerevisiae* (syn. *S. pastorianus*) was re-pitched eight times, the concentration of petite mutants in the yeast culture reached 12 % (Jenkins *et al.*, 2009).

*S. cerevisiae* was re-pitched, the levels of petite mutations in the pitching yeast did not increase correspondingly and the highest frequency of petites reached was 0.55 % (Lawrence *et al.*, 2012). Both studies were undertaken on industrial samples and the dissimilarities between the two studies was suspected to be due to yeast storage and handling stresses (Lawrence *et al.*, 2012).

It has been postulated that high levels of petite mutations present in fermentations may cause problems (Ernandes *et al.*, 1993; Lodolo *et al.*, 2008). Van Zandycke and colleagues found that petite cells showed reduced flocculation using the improved Helm's test, when compared to its respiratory competent strain (Van Zandycke *et al.*, 2002). RD yeasts were also shown to ferment at a slower rate than respiratory sufficient (RS) cells (Šilhánková *et al.*, 1970a; Ernandes *et al.*, 1993).

Several authors have found that RD cells cause off flavours in the beer (Šilhánková *et al.*, 1970a; Ernandes *et al.*, 1993; Lodolo *et al.*, 2008). Šilhánková and colleagues investigated flavours in beer that were fermented with cultures of yeast where approximately 100 % of the fermented culture had mutated cells (Šilhánková *et al.*, 1970a). The results suggested the beer was more aromatic and contained lower levels of higher aliphatic alcohols (Šilhánková *et al.*, 1970a). Conversely, another study demonstrated that beer fermented with an RD strain produced more isobutanol and isoamyl alcohol in the beer than its parent strain (Ernandes *et al.*, 1993). The diacetyl levels were also higher in the beer produced with the RD strain. Lastly, the results showed that the mutated strain did not accumulate as much biomass as the RS strain (Ernandes *et al.*, 1993).

Previous publications provided an initial view of the complexity of how approximately 100 % of petite mutated cultures of yeast affect beer and fermentation. It is more important to understand, however, the effect that petites have on fermentation and final product at the frequencies of their natural occurrence.

This study investigated the extent to which petites contribute to the fermentation of beer and the flavours produced when beer is fermented with low levels of induced petite mutations in the inoculated yeast. Additionally, the impact of how low levels of petites effect the density attenuation and the yeast in suspension trends during fermentation were investigated. The levels of petites were investigated in fermentations without serial re-pitching and thus the ASBC Yeast-14 method was suitable for this study.



## 6.2 Experimental Design

A preliminary experiment was completed to determine the mutation rate of two different yeast strains (SMA and W34/70) when exposed to varying concentrations of ethidium bromide. Ethidium bromide has been used widely to induce petite mutations in yeast cells (De Nobrega Bastos and Mahler, 1976; Hall *et al.*, 1976; Mattick and Nagley, 1977). Ethidium bromide has a high affinity for mtDNA (Sayas *et al.*, 2015). Therefore, with enough exposure to the ethidium bromide the mutagenic capabilities of the dye render the cell ‘petite’ (Slonimski *et al.*, 1968). Additionally, petite mutants may recover their respiratory competence once becoming induced. Longer exposure to the ethidium bromide, however, produces stable petite mutations (Wheelis *et al.*, 1975). For these reasons, induction of the mutations during the propagation step was chosen.

A loopful of yeast was taken from a YEPD agar slope and inoculated into 50 mL of YEPD broth. The yeast was cultured for 24 hours in a shaking incubator (30 °C, 100 rpm). The yeast was centrifuged to pellet, the supernatant was discarded, and the pellet was suspended in distilled water three times. The yeast was pitched at  $1.5 \times 10^7$  viable cells/mL into YEPD broth with varying levels of ethidium bromide concentrations for different lengths of time (Table 6.1). Upon completion of the treatment, the petite mutation frequency was determined by a dual plating method with glycerol and glucose (Section 2.12)

Yeast was propagated for the primary experiment by the propagation section in the ASBC Yeast-14 method (Section 2.3.1). This yielded a rate of 3.66 % petites testing by the TTC overlay technique (Section 2.11). A modification of the propagation step was added to produce higher frequencies of petite mutations. On the second day of propagation, the yeast cells were washed and inoculated into 100 mL of YEPD broth that contained 20 µg/mL of ethidium bromide.

Prior to the end of yeast propagation, previously frozen wort from batch #1501 (Section 2.2.2) was prepared for the start of fermentation. The fermentations were set up and monitored by ASBC Yeast-14 method as described in an earlier chapter (Section 2.3.2). At the beginning of fermentation, 10 samples of the inoculated wort were taken for petite mutation quantification (Section 2.11) (see Appendix A). At the end of fermentation, three of the fermented tubes were centrifuged (3000 x g, 3 minutes). The supernatant was stored at -20 °C in 50 mL centrifuge tubes until sampled for volatile flavours (Section 2.9). The changes in flavour compounds were analysed using linear regression.

### 6.3 Results

The preliminary experiment showed that yeast strain SMA yielded the highest percentage of petites of 92 % when the culture was incubated for 24 hours with 20 µg/mL of ethidium bromide (Table 6.1). The other yeast strain tested, W34/70, yielded 82 % petites in the culture after the same incubation time and ethidium bromide treatment.

**Table 6.1.** Preliminary experiment to determine the petite mutation rates of strains SMA and W34/70. Cells were incubated at stated concentration of ethidium bromide in YEPD broth at 30 °C and 100 rpm. After the stated time interval the cells were plated onto YEPD and YEPGLy plates to determine the percentage of petites.

Yeast Strain	[EtBr] (µg/mL)	Incubation time (h)	Cell count		% Petites
			YEPD	YPGLy	
SMA	10	1	389	379	3
		4	TNTC*	TNTC	ND**
		24	398	237	40
SMA	20	1	708	628	11
		4	TNTC	TNTC	ND
		24	441	34	92
W34/70	10	1	688	704	-2
		4	TNTC	TNTC	ND
		24	156	122	22
W34/70	20	1	620	652	-5
		4	TNTC	TNTC	ND
		24	558	98	82

\*TNTC = Too numerous to count

\*\* ND = No data

The viabilities of each yeast culture were determined prior to mixing to achieve the desired levels of petite mutations in the pitched yeast (Table 6.2). The viabilities were determined by methylene blue (Section 2.5.2) and remained above 98% viable for all cultures.

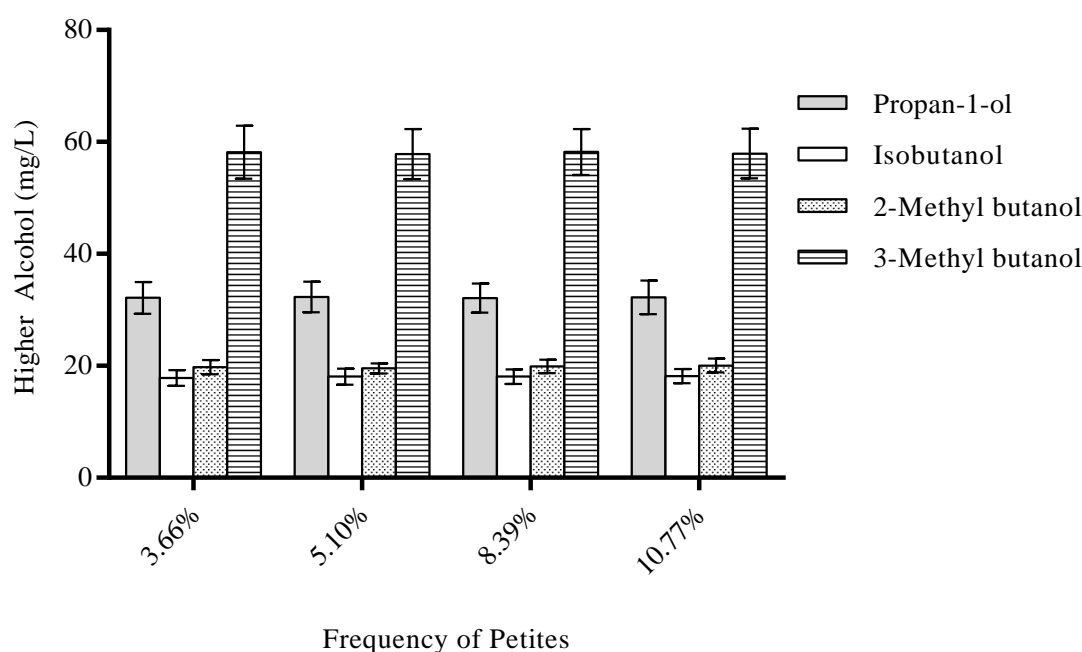
**Table 6.2.** Viabilities of yeast cultures used after propagation and prior to blending for pitching. Means represent duplicate counts with standard deviation where n = 2.

	3.66 % Petite Fermentation		5.10 % Petite Fermentation		8.39 % Petite Fermentation		10.77 % Petite Fermentation	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Grandes	99.66	0.01	99.56	0.23	99.49	0.28	99.56	0.62
Petites	ND	ND	98.40	0.24	99.44	0.23	98.63	0.38

In the primary experiment, volatile flavour compounds were analysed at the end of all fermentations. The higher alcohols analysed by GC-MS were propan-1-ol, 3-

methanol, 2-methyl butanol, and isobutanol. The higher alcohol 3-methyl butanol was present in the highest quantities at  $58.02 \pm 3.79$  mg/L. No significant differences were found between petite mutation levels and the presence of higher alcohols post-fermentation (Figure 6.1). The levels of higher alcohols in each fermentation remained unchanged, regardless of the levels of petite mutations present during the fermentation.

Acetaldehyde, ethyl acetate, isobutyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, isoamyl acetate, 2,3-butanedione, and 2,3-pentanedione showed linear correlations with the increasing frequency of petite mutations in the pitching yeast (Table 6.3). Of these positive linear correlations, ethyl acetate showed the largest rise in the flavour compound levels related to the petite mutation frequency. The largest rise was determined by the slope ( $m$ ) of the linear regression (Figure 6.2). Acetone was not observed to have a significant linear correlation to the increasing percentage of petites (Figure 6.3).

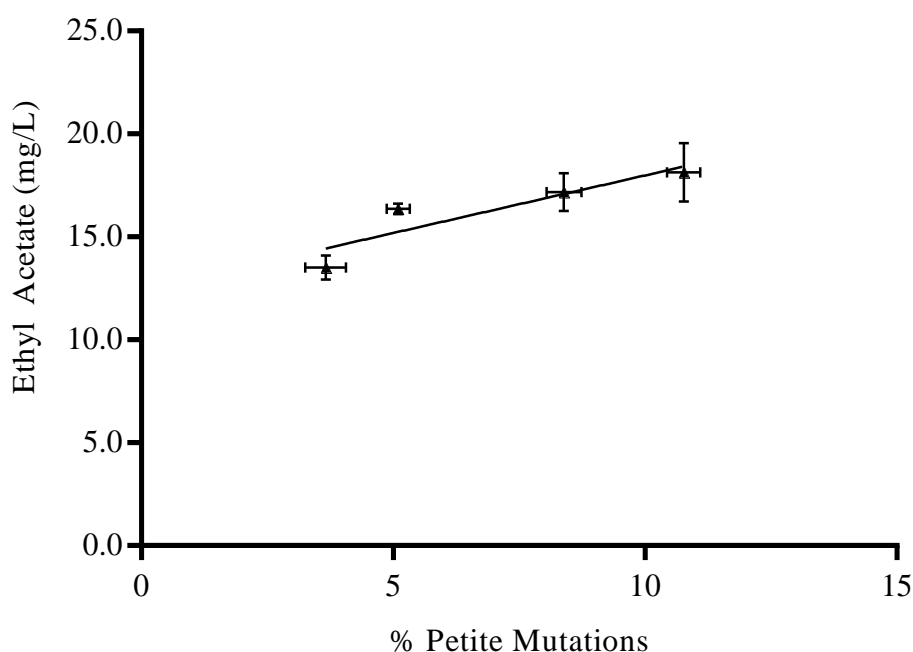


**Figure 6.1.** Higher Alcohols present in beer post primary fermentation when fermented with varying levels of petite mutations in the pitched yeast.

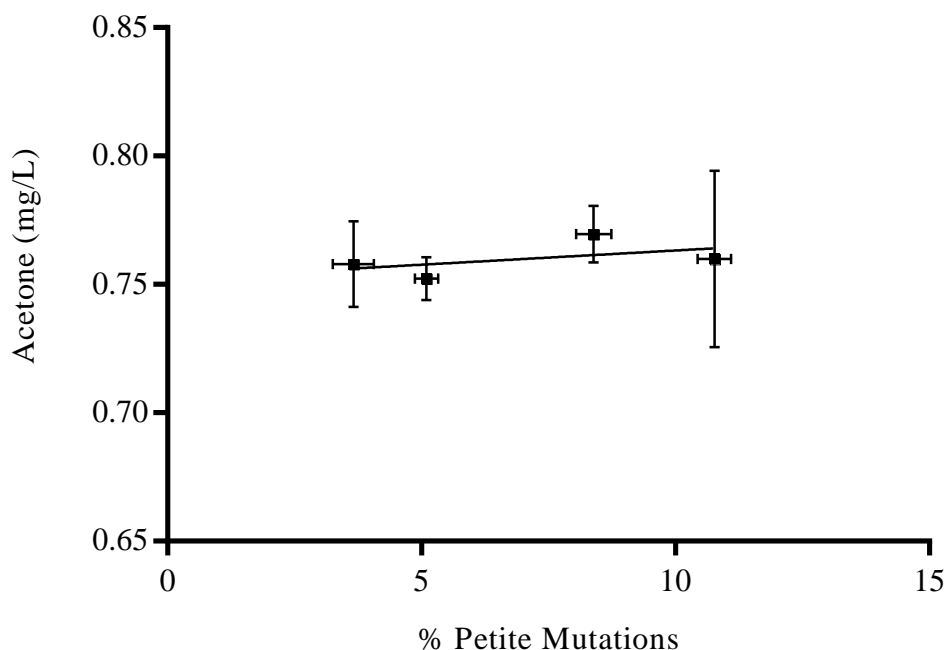
**Table 6.3.** Summary of flavour compounds analysed by linear regression as the percentage of petite mutation in the pitching yeast increased from 3.66 % to 10.77 %. The table includes: the slope (m), y-intercept (b), the goodness of fit for the linear regression ( $R^2$ ).

Flavour Compound	Slope (m)	Y intercept (b)	$R^2$	Significance <sup>a</sup>
Acetaldehyde	0.1667	10.28	0.9431	****
Acetone	0.001104	0.7522	0.02977	ns
Ethyl acetate	0.5597	12.39	0.6895	***
Isobutyl acetate	0.001563	0.03119	0.4421	**
Ethyl butyrate	0.002155	0.04086	0.9552	****
Propan-1-ol	0.0002408	32.19	8.5E-08	ns
Isobutanol	0.03515	17.79	0.00768	ns
Isoamyl acetate	0.05882	1.051	0.844	****
2-Methyl butanol	0.0539	19.46	0.02397	ns
3-Methyl butanol	-0.006955	58.07	2.8E-05	ns
Ethyl hexanoate	0.004165	0.07743	0.8291	****
Ethyl octanoate	0.01449	0.04193	0.9449	****
2,3-Butanedione	0.005108	0.07111	0.4296	*
2,3-Pentanedione	0.00687	0.1116	0.3465	*

<sup>a</sup> ns = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$



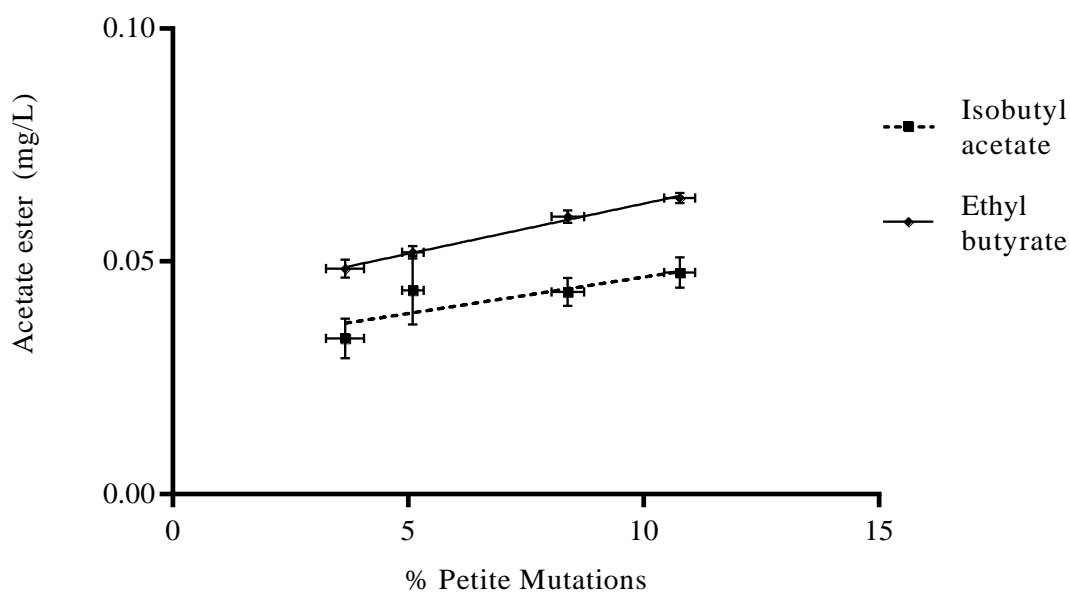
**Figure 6.2.** Linear correlations for ethyl acetate concentrations post primary fermentation as the percentage of petite mutations in the pitched yeast increased. Each point represents triplicate samples. The coefficient of determination is noted in Table 6.3.



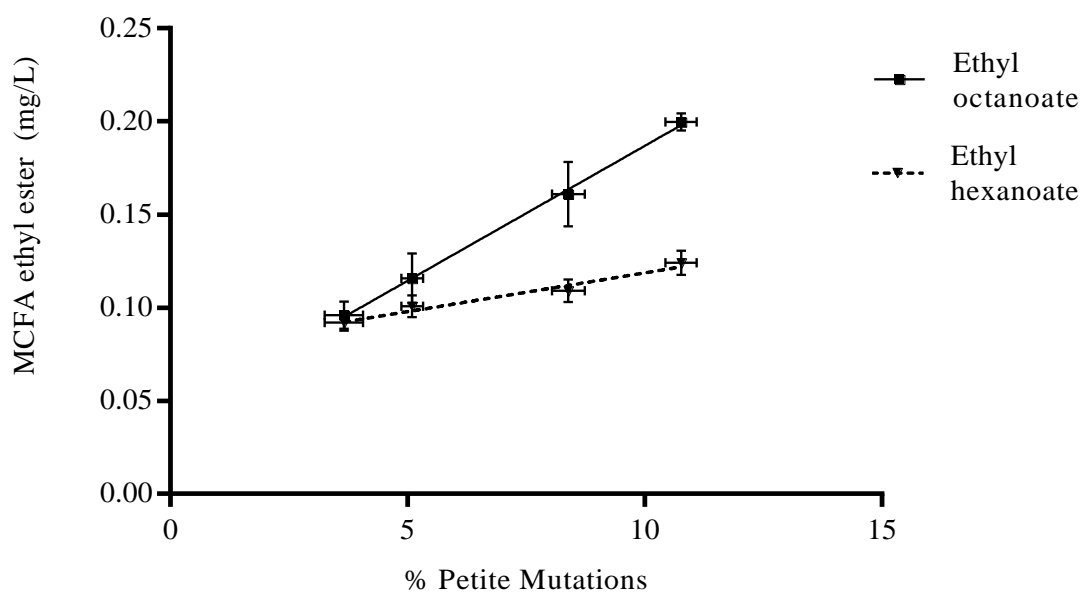
**Figure 6.3.** Levels of acetone concentrations post primary fermentation as the percentage of petite mutations in the pitching yeast increases. Each point represents triplicate samples. The coefficient of determination is noted in Table 6.3.

Acetaldehyde, ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate had strong or very strong linear correlations to the petite mutation frequency in the pitched yeast ( $r^2 = 0.6895 - 0.9552$ ). Isobutyl acetate and 2,3-butanedione had moderate positive correlations to the frequencies of petites in the culture ( $r^2 = 0.4296 - 0.4421$ ). Finally, 2,3-pentanedione had a weak positive correlation ( $r^2 = 0.3465$ ), as described by Evans (1996). The positive linear correlations are shown with increasing petite mutant concentration for ethyl butyrate and isobutyl acetate (Figure 6.4), ethyl hexanoate and ethyl octanoate (Figure 6.5), and isoamyl acetate (Figure 6.6). The increasing trend of the concentrations of the vicinal diketones were shown compared to the frequency of petites in the pitched yeast (Figure 6.7).

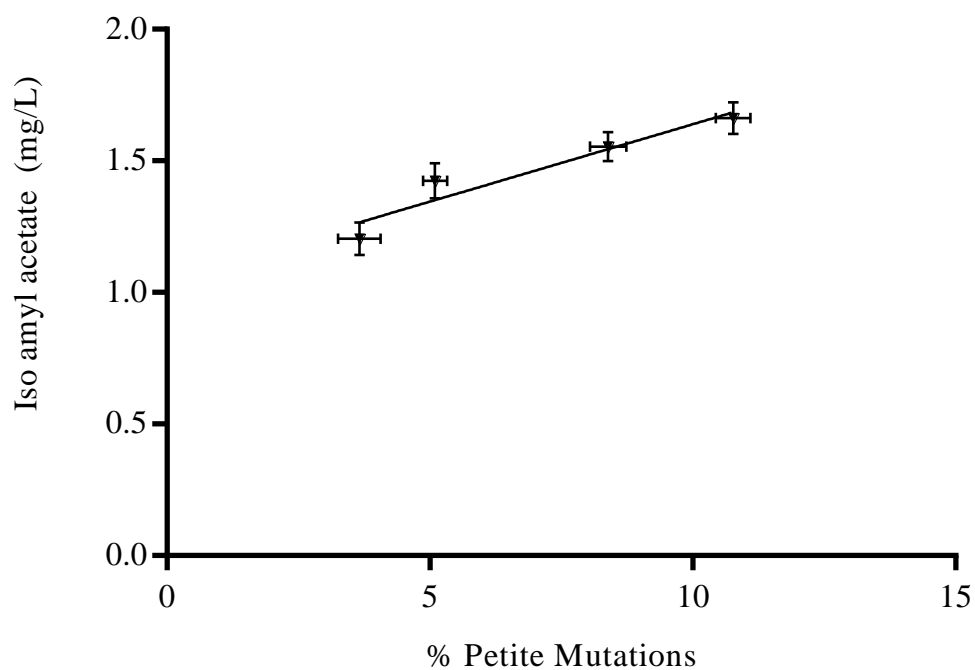
Acetaldehyde was measured however the results were not used in this analysis. The correlation for the calibration curve used to determine the acetaldehyde concentration was  $R^2 = 0.3$ . This correlation was too low for an accurate quantification of the actual concentrations of acetaldehyde in the beer.



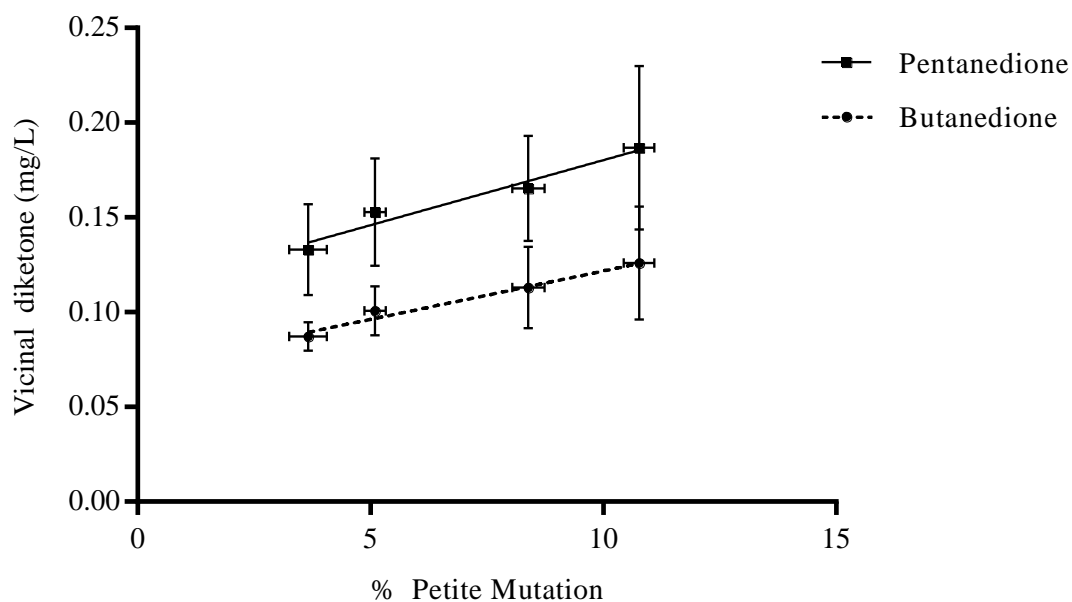
**Figure 6.4.** Linear correlations for acetate ester concentrations, isobutyl acetate and ethyl butyrate, post primary fermentation as the percentage of petite mutations in the pitched yeast increased. Each point represents triplicate samples. The coefficient of determination is noted in Table 6.3.



**Figure 6.5.** Linear correlations for MCFA ethyl ester concentrations, ethyl hexanoate and ethyl octanoate, post primary fermentation as the percentage of petite mutations in the pitched yeast increased. Each point represents triplicate samples. The coefficient of determination is noted in Table 6.3.



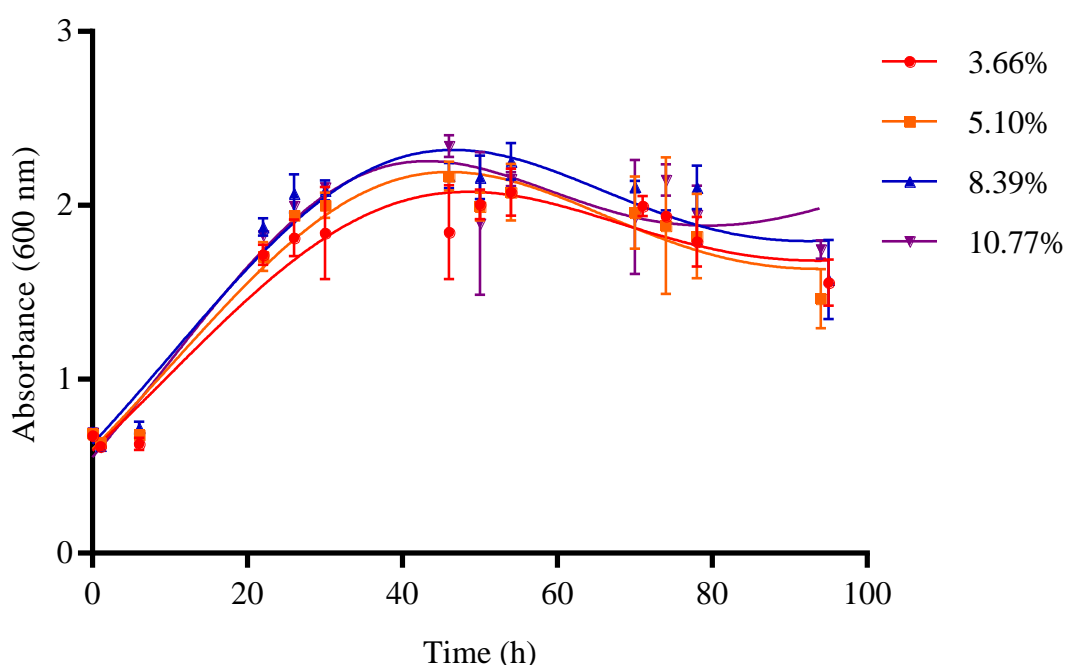
**Figure 6.6.** Linear correlations for isoamyl acetate concentrations post primary fermentation as the percentage of petite mutations in the pitched yeast increased. Each point represents triplicate samples. The coefficient of determination is noted in Table 6.3.



**Figure 6.7.** Linear correlations for vicinal diketone concentrations, 2,3-butanedione and 2,3-pentanedione, post primary fermentation as the percentage of petite mutations in the pitched yeast increased. Each point represents triplicate samples. The coefficient of determination is noted in Table 6.3.

The absorbance was measured systematically as per ASBC Yeast-14 during fermentation and the values of which acted as a representation of the yeast in suspension (Figure 6.8). Results showed no significant difference ( $p < 0.05$ ) in yeast in suspension behaviour at the levels of petite mutated yeasts tested. Therefore, it can be said that regardless of the petite mutation frequency in the culture, the yeast growth during fermentation was unchanged.

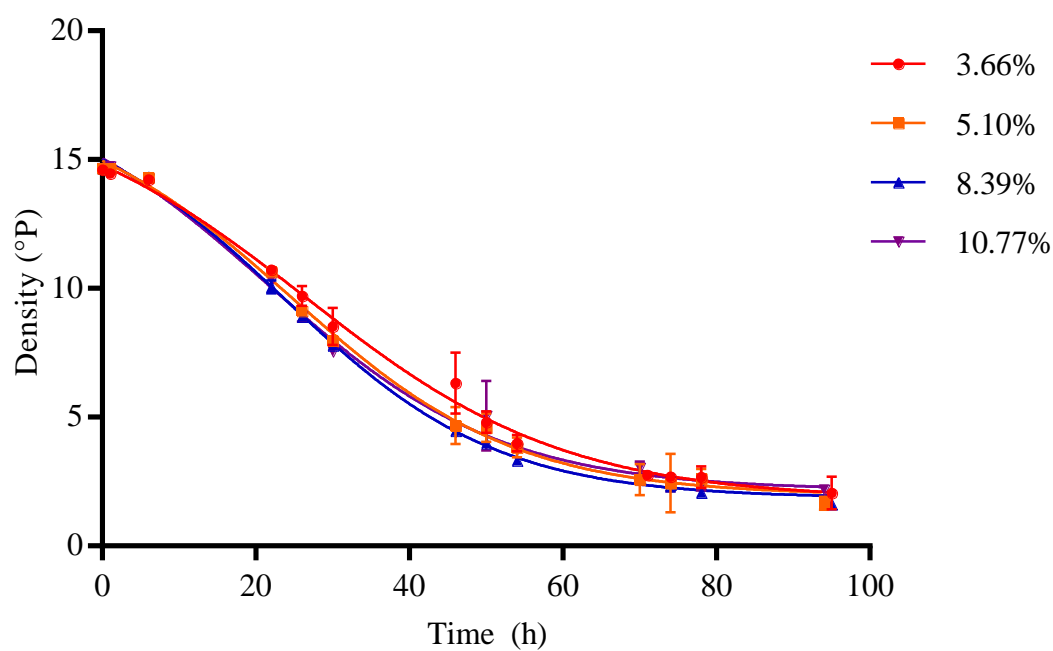
The density attenuation for all fermentations was modelled by the four-parameter non-linear regression (Figure 6.9). None of the parameters in the nonlinear regression were determined to have significant difference between varying levels of petites in the pitched yeast ( $p > 0.05$ ).



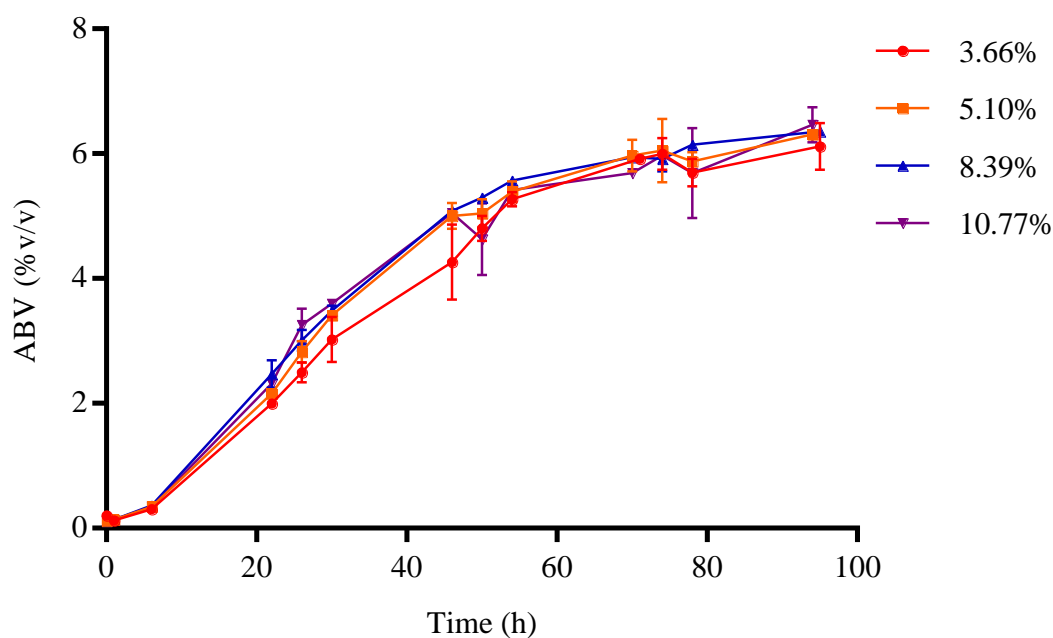
**Figure 6.8.** Yeast in suspension trends as the fermentations progressed from start to finish with varying levels of petites: 3.66 % (red), 5.10 % (orange), 8.39 % (blue), and 10.77 % (purple). Trends were analysed using the tilted Gaussian fit.

Additionally, the alcohol by volume (ABV) was monitored through the fermentation (Figure 6.10). By the end of fermentation, the treatment with the lowest ABV was with 3.66 % petites at  $6.11 \pm 0.37$  %. The petite treatment with the highest ABV at the end was with 10.77 % petites at  $6.46 \pm 0.28$  %. A one-way ANOVA showed that the differences in levels of final ABV levels were not significantly different between the four fermentations at the end of the fermentation.





**Figure 6.9.** The density attenuation during the four fermentations with varying levels of petites in the pitching yeast.



**Figure 6.10.** Alcohol by volume (ABV) for fermentations containing various levels of petite mutations in the pitched yeast.

## 6.4 Discussion

### 6.4.1 Preliminary Mutation Rates

Incubating the cells with 10 and 20 µg/mL of ethidium bromide for 1 hour was not sufficient to mutate the cells. Plating after four hour exposure, it was found that there were too many cells to count the plates. Also, under visual inspection, however, both plates showed similar growth suggesting that four hours was too short for induction of petite mutations in these conditions. A large frequency of petites was induced with both strains when they were propagated with 20 µg/mL of ethidium bromide for 24 hours. Strain SMA showed the greater susceptibility to induction and was utilized for the primary study. After 24 hours of incubation with 20 µg/mL in YEPD broth, the frequency of petites in the culture for strain SMA and strain W34/70 were 92 and 82 % respectively.

### 6.4.2 Flavours in the Beer

#### *Higher Alcohols*

Previous studies examined the levels of higher alcohols in beer fermented with high levels (100 %) of petite mutations in the pitched yeast. One study showed increased levels of isobutanol, isoamyl alcohol, and propan-1-ol when fermenting with a 100 % RD culture in comparison to fermenting with its respiratory sufficient (RS) culture (Ernandes *et al.*, 1993). Morrison and Suggett (1983) also investigated flavour profiles post fermentation with a culture from a singular petite isolate compared its RS type. The later study found isobutanol and isoamyl alcohol levels were increased, however, propan-1-ol levels were decreased (Ernandes *et al.*, 1993). Contrary to both of those studies, a third study investigated these three higher alcohols and found decreased levels in fermentations with 100 % RD cultures (Šilhánková *et al.*, 1970a). Thus, the impact that high levels of petite mutations have on higher alcohol levels does not seem universal. The differences observed could be due to several factors such as yeast strain, petite mutation type, and/or wort composition. The current study found with increasing levels of petites (< 10.77 %) in the pitching yeast the levels of propan-1-ol, isobutanol, 2-methyl butanol and 3-methyl butanol were unchanged. The results from the current study suggest when petites are present in low concentrations, their over or underproduction of higher alcohols in fermentations (Šilhánková *et al.*, 1970a; Morrison and Suggett, 1983; Ernandes *et al.*, 1993) may be concealed by the normal brewery variation in higher alcohol production.

## *Esters*

Two prior studies discussed above also investigated ester levels in beer fermented with high levels of petites (Morrison and Suggett, 1983; Ernandes *et al.*, 1993). Differing from the current study, Morrison and Suggett (1983) showed the concentrations of six esters in beer fermented with a RD strain, were lower than the concentrations of the esters in beer fermented with its RS strain. Later, Ernandes *et al.* (1993) found that levels of isoamyl acetate and ethyl acetate post fermentation were decreased for beer fermented with a petite mutant strain when compared to RS type (Ernandes *et al.*, 1993) while the ethyl octanoate and phenyl ethyl acetate were increased. Considering that esters are influenced by several factors in fermentation, there may be several reasons for the discrepancy between studies such as yeast strain, petite mutation type, wort composition, fermentation temperature, and/or wort aeration rates for examples.

There are two main types of esters produced by yeast in fermenting beer: acetate esters and medium chain fatty acid ethyl esters (MCFA). MCFA ethyl esters are formed by a condensation reaction with acyl-CoA and ethanol (Pires *et al.*, 2014), and the enzymes involved in the synthesis of these are coded by Eeb1 and Eht1 genes (Saerens *et al.*, 2006). The MCFA esters in the current study analysed were ethyl hexanoate and ethyl octanoate. The findings presented here for ethyl octanoate agree with those of Ernandes *et al.* (1993) that found an increase in ethyl octanoate in the RD strain when compared to its defined RS strain. However, the findings disagree with Morrison and Suggett (1983), who found a decrease in both ethyl octanoate and ethyl hexanoate.

Acetate esters are anabolized by the enzyme alcohol acetyltransferase (AAT) coded by ATF genes (Pires *et al.*, 2014). These esters are formed by a condensation reaction between acetyl CoA and alcohol (Pires *et al.*, 2014). The acetate esters investigated in this study were ethyl acetate, isoamyl acetate, ethyl butyrate, and isobutyl acetate, which increased with respect to increased petite mutations in the pitched yeast.

One plausible cause for observing the increase in acetate esters and MCFA ethyl esters could be due to excess acetyl CoA from disruption in other pathways that also utilize acetyl CoA. The compound acetyl CoA is utilized in the TCA cycle and is involved in the synthesis of lipids, nucleic acids, amino acids, and esters (Peddie, 1990; Pires and Brányik, 2015). Petites are unable to synthesize fatty acids as they lack functional mitochondria necessary for lipid synthesis (Lodolo *et al.*, 2008).

Additionally, in petite mutant cells, the TCA cycle genes are downregulated (Liu and Butow, 1999), thus potentially increasing the pool of acetyl CoA available for ester synthesis. Supporting this hypothesis, a past study showed that once lipid synthesis during normal fermentation ceases, acetate ester synthesis rates increase (Thurston, Quain and Tubb, 1982). In petite mutated cells lipid synthesis is disrupted and the higher levels of esters observed in the current study from fermentation with higher levels of petites could result from ester synthesis commencing earlier in fermentation for RD yeasts than its RS type.

#### *Vicinal Diketones*

Levels of 2,3-pentanedione and 2,3-butanedione increased in concentration corresponding with the level of petite mutations. The samples for all flavour compounds were taken near the end of fermentation at hour 78. Nearing the end of fermentation, vicinal diketone levels are dynamic because the yeast are absorbing the vicinal diketones in the medium and converting them to diols (Krogerus and Gibson, 2013). Any difference in fermentation progression between fermentations at hour 78 may contribute to some variation observed between samples. In general, published literature shows elevated vicinal diketone levels are associated with high levels (approximately 100 %) of petite mutations in the pitched yeast (Morrison and Suggett, 1983; Ernandes *et al.*, 1993). Dasari and Kölling (2011), provided a thorough study on petite mutants and their production of  $\alpha$ -acetolactate in fermentation. These authors showed that the key enzyme (Ilv2) in  $\alpha$ -acetolactate synthesis, which normally resides in the mitochondria, accumulated in the cytosol yeasts (Dasari and Kölling, 2011). Their data strongly suggested that the enzyme accumulation in the cytosol was linked to the elevated levels of 2,3-butanedione observed in the beer when fermented with high levels petites (Dasari and Kölling, 2011). The study hypothesized that the transport of  $\alpha$ -acetolactate from the cell into the fermenting medium was easier when it was synthesized in the cytosol rather than the mitochondria because there were fewer cell membranes for the synthesized  $\alpha$ -acetolactate to cross (Dasari and Kölling, 2011)

The current study contributes to current knowledge and showed that even with low levels of petites, the 2,3-butanedione and 2,3-pentanedione levels were increased. Demonstrating that even in fermentations that contained low frequencies of petite mutations in the pitched yeast, increased levels of vicinal diketones may be a concern.

### Overall flavour impact

Flavour thresholds vary depending on several factors, such as the individual flavour compound, beer style and human sensitivity (Schönberger and Kostecky, 2011) making it difficult to determine when the increase in flavour compound would be noticeable in the beer. Even though in this experiment the levels of esters increased with increasing levels of petites in the pitched yeast, it does not necessarily indicate that flavour changes between the low levels of petites studied would be detectible to the consumer. From the results, the differences in ester and VDK concentrations produced from 0 to 5 % increase and 0 to 10 % increase of petites in the experiment were calculated (Table 6.4) and compared to published flavour thresholds for the compounds. Using this table, it is unlikely that the change in flavour compounds produced would be detectible to the consumer because they do not surpass the documented flavour thresholds. Even though some of the flavour compounds were present in levels that are passed the flavour threshold, it is only of importance if the differences in esters or VDKs produced with the varying low concentrations of petites were detectible. This is suggested to be unlikely because the increases in esters and VDKs observed were lower than the flavour thresholds.

**Table 6.4.** Using the linear regression of each flavour compound studied in the experiment, the difference between 0 and 5 % petites, and 0 and 10 % petites were calculated from Table 6.3.

Flavour Compound	$\Delta$ [flavour compound] (mg/L) from 0 % and 5 % petites	$\Delta$ [flavour compound] (mg/L) from 0 % and 10 % petites	Threshold (mg/L)
Ethyl acetate	2.80	5.60	25-30 (Pires and Brányik, 2015)
Isobutyl acetate	0.0078	0.016	1.6 (Renger <i>et al.</i> , 1992)
Ethyl butyrate	0.011	0.022	0.3 (Aroxa, n.d.)
Isoamyl acetate	0.29	0.59	1.2-2.0 (Pires and Brányik, 2015)
Ethyl hexanoate	0.021	0.042	0.2-0.23 (Pires and Brányik, 2015)
Ethyl octanoate	0.072	0.15	0.9-1.0 (Pires and Brányik, 2015)
2,3-Butanedione	0.026	0.051	0.1-0.15 (Pires and Brányik, 2015)
2,3-Pentanedione	0.034	0.069	0.9-1.0 (Pires and Brányik, 2015)

### 6.4.3 Density Attenuation

Previously published literature illustrated that fermentations with petite mutated cells fermented slower than their parent strain (Šilhánková *et al.*, 1970a; Ernandes *et al.*, 1993). In the experimental work presented here, the impact that petites had on density attenuation at high frequencies was determined to be undetectable when low frequencies of petites were present. Even though petite mutations ferment at a slower rate than their grande-type, when the petite mutants were present in low quantities, no difference in fermentation speed (B) or time for the fermentation to reach the midpoint (M) was detectible. Two previously studies (Šilhánková *et al.*, 1970a; Ernandes *et al.*, 1993) both found that the petite strains did not reach final gravity compared to their respiratory competent genotype. In the current study, no significant difference in end gravity ( $P_e$ ) was noted between all fermentations. This showed low levels of petite mutations did not have an impact on different fermentation parameters: final density, the midpoint, and speed of fermentation.

### 6.4.4 Yeast in Suspension

Two previously published studies demonstrated that during fermentation, a respiratory deficient strain produced less biomass compared its RS strain (Šilhánková *et al.*, 1970a; Ernandes *et al.*, 1993). This trend was not observed to be consistent in this current study. The small-scale fermentations undertaken demonstrated no significant difference ( $p > 0.05$ ) between the four fermentations with respect to the yeast in suspension profile. This result was unexpected as the petite mutant is considered a weak competitor to the RS strain, due to slower growth (Šilhánková *et al.*, 1970b). Therefore, one might expect a decreased peak with respect to yeast in suspension trends, as petites levels increased during the fermentation, but this was not observed. Most cells present in fermentation were RS with a small percentage of RD cells. Even though petite mutations exhibit slower growth, the normal fermenting capacity of the normal cells in fermentation seemed to compensate for the petite mutations. In the work presented a petite population of up to 10.77 %, did not have an impact on the overall yeast growth in fermentations. This observation was proposed to be from the slower growth of the respiratory deficient strain, therefore leaving more nutrients available in the fermenting beer for growth of the RS strain.

The absorbance trends towards the end of the fermentation indicate yeast flocculation patterns. The flocculation patterns are observed by the decline in absorbance. The ASBC method, Yeast-14 method was originally designed to investigate

whether malt was susceptible to premature yeast flocculation (MacIntosh *et al.*, 2012). This analysis was done by using the absorbance (600 nm) to determine flocculation patterns of the yeast during fermentation. The modelled yeast in suspension trends were not significantly different. This finding suggests that the flocculation trends between the four fermentations were not affected by the presence of petites from 3.66 % up to 10.77 %. The lack of impact on fermentation profile could be surprising given that the literature shows that petites have altered cell walls, however these results suggest those effects are not apparent on the yeast in suspension profiles when at the low frequencies.

For all treatments, a large fraction of the total yeast at the end of fermentation was found to be still in suspension. It is possible that RD mutants could become a significant factor post fermentation. To gain more of an understanding on the flocculation trends in fermentations with low levels of petites, the yeast in suspension would need to be monitored past the time it took for the fermentation to reach its final gravity. However, the yeast in suspension trends shown here demonstrate that the physical flocculation trends in the primary fermentation are not impacted by low levels of petite mutations in the pitched culture.

## **Chapter 7 – The Effect of Yeast Storage Time Between Fermentations on Extended Serial Re-pitching**

### **7.1 Introduction**

Few industrial breweries continuously start the subsequent re-pitched fermentation immediately after the yeast has been cropped from the previous fermentation. Therefore, storage of yeast between fermentations is necessary (Section 1.2.6).

During storage, the cell's metabolism is slowed by reducing the temperature. Typically, yeast slurry storage temperatures are held between 3 °C and 4 °C (Somani *et al.*, 2012). However, researchers have identified one strain (W34/70) that demonstrated very little physiological differences between storage at 4 °C and 10 °C (Somani *et al.*, 2012). Upon analysing the glucose induced proton efflux (GIPE), which indicates the sugar utilization from the environment, the yeast stored at 10 °C had a higher GIPE than the yeast stored at 4 °C. Additionally, no significant difference between the viabilities of the two yeast cultures was reported (Somani *et al.*, 2012). During starvation, the yeast utilizes glycogen as an energy source (Lillie and Pringle, 1980). Cells possess two pools of glycogen: in the cell wall and intracellular stores (Deshpande *et al.*, 2011). The glycogen concentration in the cell wall is dynamic during fermentation and sensitive to changes in sugar concentrations within the wort. The intracellular glycogen was found to remain constant.

The impacts of stirring the yeast culture during storage on cell and fermentation quality have been discussed in previously published literature (McCaig and Bendiak, 1985a; Sall *et al.*, 1988). McCaig and Bendiak (1985a) found that between still, constant agitation, and periodic agitation of yeast during storage, the cells treated with constant agitation had the greatest amount of deterioration. The deterioration was measured by the change in glycogen content and % viability of the cells (McCaig and Bendiak, 1985a). In their study, the observations for cellular deterioration between yeast stored still and yeast stored with periodic agitation, were similar. After these treatments, fermentation was set up and the fermentations' density attenuations, yeast in suspension trends, 2,3-butanedione levels, and alcohol production trends were monitored. These fermentation characteristics were similar for the yeast stored with periodic agitation and the yeast stored static. The yeast stored with constant agitation exhibited the poorest fermentation profiles based on those characteristics (McCaig and Bendiak, 1985a). Sall and colleagues (1988) raised the observation that in the study completed by McCaig and



Bendiak (1985a), oxygen was not eliminated in the yeast storage treatments that were agitated. These authors performed a similar study, and found only a small decrease in the glycogen levels in the cell during stirred storage (Sall *et al.*, 1988). This observation showed that agitation was not as detrimental to yeast glycogen stores, as previously thought. Sall and colleagues (1988), however, did not have a controlled yeast storage sample with the yeast being stored still.

Later, Rhymes and Smart (2001) investigated the impacts of yeast storage on ale yeast flocculation and cell wall properties. In this study, starved and non-starved yeast cells were stored either still or under agitation for various lengths of time (Rhymes and Smart, 2001). The cells under agitation in this study, were also exposed to oxygen. The results showed that overall the yeast cell wall properties and flocculation characteristics were affected by starvation, duration, temperature, and agitation conditions during yeast storage (Rhymes and Smart, 2001).

No studies have been identified that examined the accumulated effect of storage time on fermentation properties. Where most brewers are employing serial re-pitching techniques, it is important to understand how repeated storage times effect the entire re-pitching process. This study investigated the accumulated effects of varying storage times on eight serial re-pitched fermentations. The different storage times between fermentations were 18, 42, and 66 hours. It was hypothesized that the glycogen and trehalose levels in yeast for the 66 hour yeast storage time between fermentations would be lower than 18 and 42 hour yeast storage. It was hypothesized further, that because of the lower glycogen and trehalose levels in the yeast stored for 66 hours, the fermentation density attenuation rates would be slower and less yeast growth during fermentation observed. Lastly, it was expected that the storage temperatures would influence the vicinal diketones produced post fermentation, but not higher alcohols and esters.

## **7.2 Experimental Design**

### **7.2.1 Yeast Preparation**

For the first fermentation, yeast was propagated and prepared to inoculate 3 x 1 L fermentations in triplicate (Section 2.3.1) (Figure 7.1). The cells were counted using the Aber Countstar and inoculated into the wort at  $1.5 \times 10^7$  viable cells/mL (Section 2.5.2).

For the subsequent fermentations, the yeast slurries were removed from the fridge and the supernatant was discarded. No centrifugation was necessary to pellet the

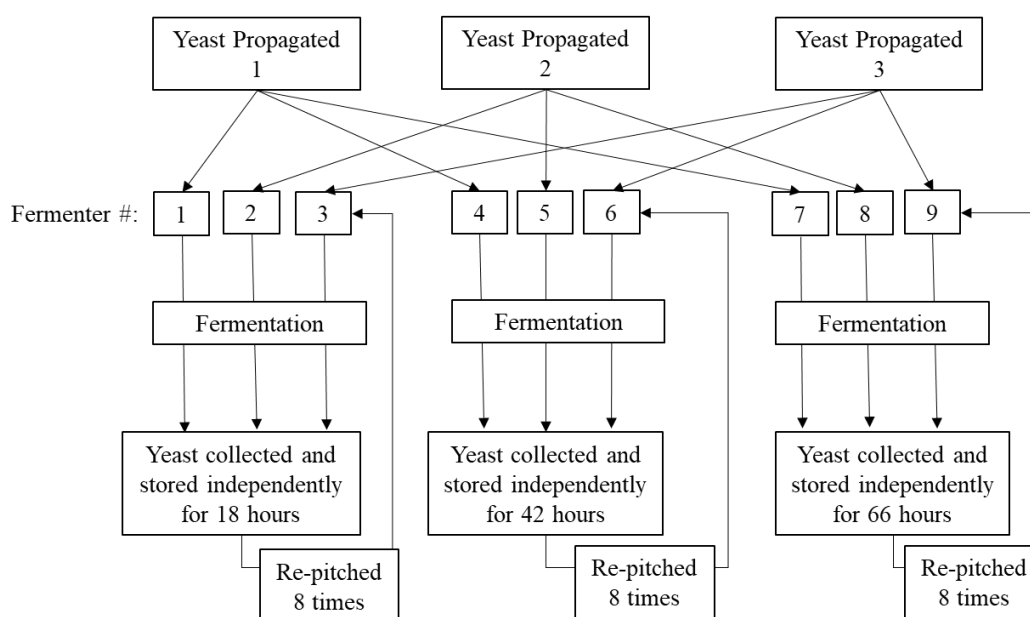
yeast cells as natural sedimentation had occurred during the storage period. The pellet was re-suspended in sterile deionized water and the slurry was centrifuged (3000 x g, 5 minutes). The cells were washed three times (Section 2.3.1). Yeast was counted and viability assessed using the Aber Countstar (Section 2.5.2).

### **7.2.2 Fermentation Preparation and Monitoring**

Nine graduated cylinder fermentations were prepared (Section 2.4) for three treatments completed in triplicate. The treatments were the yeast storage times between re-pitched fermentations of either 18, 42, or 66 hours (Figure 7.1). The final volume of the inoculated fermentations was 1000 mL. Immediately after the fermentations were pitched, the remaining slurry was placed on ice, 1 mL from the yeast slurry that remained was transferred into a 2 mL Eppendorf tube. This sample was stored at – 20 °C until analysed for glycogen and trehalose concentrations. Density (°P) and yeast in suspension by absorbance (600nm) trends were monitored during fermentation (Section 2.3.2). Finally, the collected data was modelled and analysed using non-linear regression techniques (Section 2.3.3). At hour 102 of the fermentation, a 20 mL sample was removed in duplicate from the 700 mL mark of the graduated cylinder. This sample was transferred to a 50 mL centrifuge tube. The tube was centrifuged (3 000 x g, 3 minutes) to degas and remove yeast particles from the beer. The samples were stored at – 60 °C until analysed for ester, higher alcohols, and vicinal diketones (Section 2.9).

### **7.2.3 Yeast Storage**

After the samples of beer were taken at hour 102 for flavour compound analysis, the yeast was cropped. To collect the yeast, the tip of a sterile 25 mL glass pipette was placed at the yeast sediment/beer interface. The pipette tip remained at this location and 25 mL of yeast was collected and transferred into a 50 mL sterile centrifuge tube. This was performed twice. The yeast was centrifuged (3 000 x g, 3 minutes) and the supernatant was discarded. Two more 25 mL aliquots of yeast sediment were collected and transferred into the same 50 mL centrifuge that contained cropped yeast. This sample was centrifuged again (3 000 x g, 3 minutes) and the supernatant discarded. Yeast was collected from the fermenter one more time and transferred into the centrifuge tube with cropped yeast. The yeast was collected until the yeast slurry in beer reached the 40 mL mark on the centrifuge tube. The pelleted yeast was re-suspended in the collected beer using a vortex mixer. The lid was closed tightly and sealed with parafilm. The yeast slurry was stored at 4 °C between fermentations. The yeast was stored for either 18, 42, or 66 hours (Figure 7.1).



**Figure 7.1.** Flow diagram of the experimental design for the yeast storage experiment

#### 7.2.4 Statistical Analysis

Throughout this analysis, a two-way ANOVA was the chosen statistical analysis to compare the storage treatment and the re-pitched number to the flavour compounds and the yeast storage carbohydrates. The two-way ANOVA is normally performed when there are two nominal variables. The re-pitched number, however, was not a nominal variable. By considering the treatment as a nominal variable, the approach becomes more conservative, which does not violate any statistical assumptions (Systat Software, 2009). Therefore, to determine if the variation was significant, a two-way ANOVA was performed. For all statistical analysis, a  $p$  value  $< 0.05$  was considered significant. The density attenuation and yeast in suspension trends were modelled and analysed as normal (Section 2.3.3).

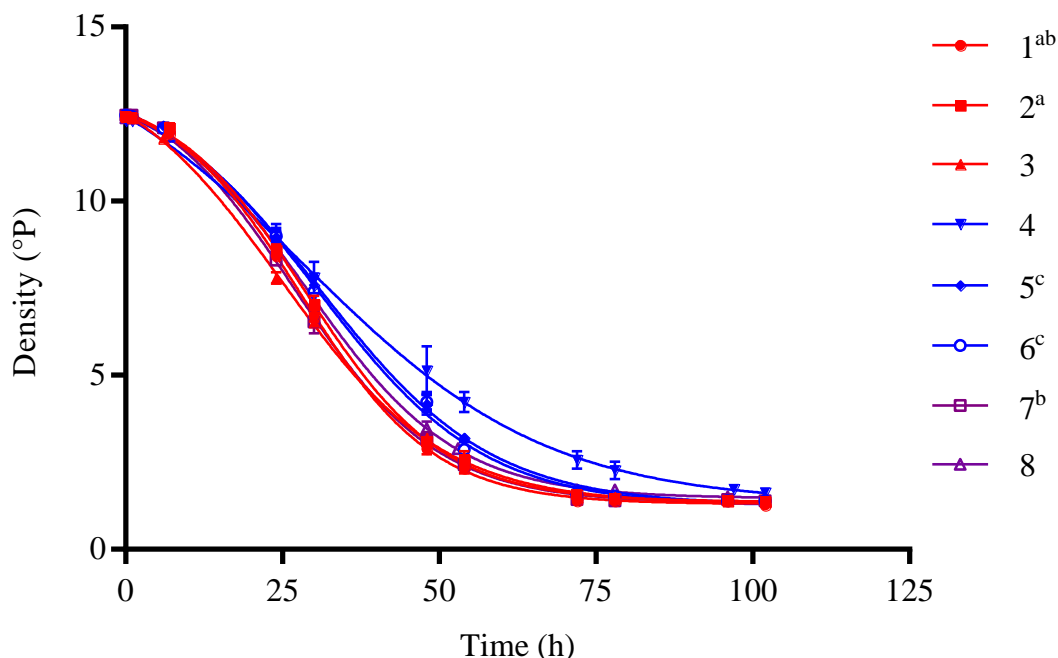
### 7.3 Results

#### 7.3.1 Physical Fermentation

The density at the start of fermentation for all storage treatments were between 12.2 and 12.5 °P. The final density measurements for all fermentations ended between 1.3 and 1.7 °P.

Density attenuation trends for eight serially re-pitched fermentations with the yeast stored for 18 hours were monitored (Figure 7.2). Fermentation four took the longest time to reach density equilibrium. The density attenuation of this fermentation was significantly different than all other fermentations. Most attenuation trends were significantly different from the other attenuation trends in the series with three

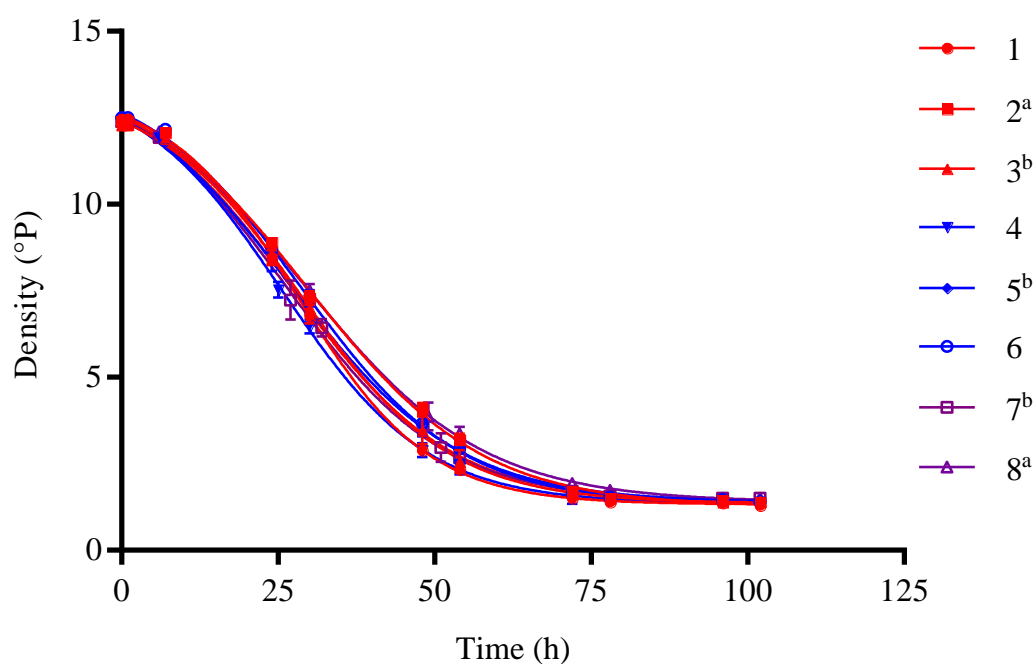
exceptions. Those exceptions were between fermentations one and two, fermentations one and seven, and fermentations five and six (Figure 7.2). Between these stated fermentations, the density attenuation rates were not significantly different.



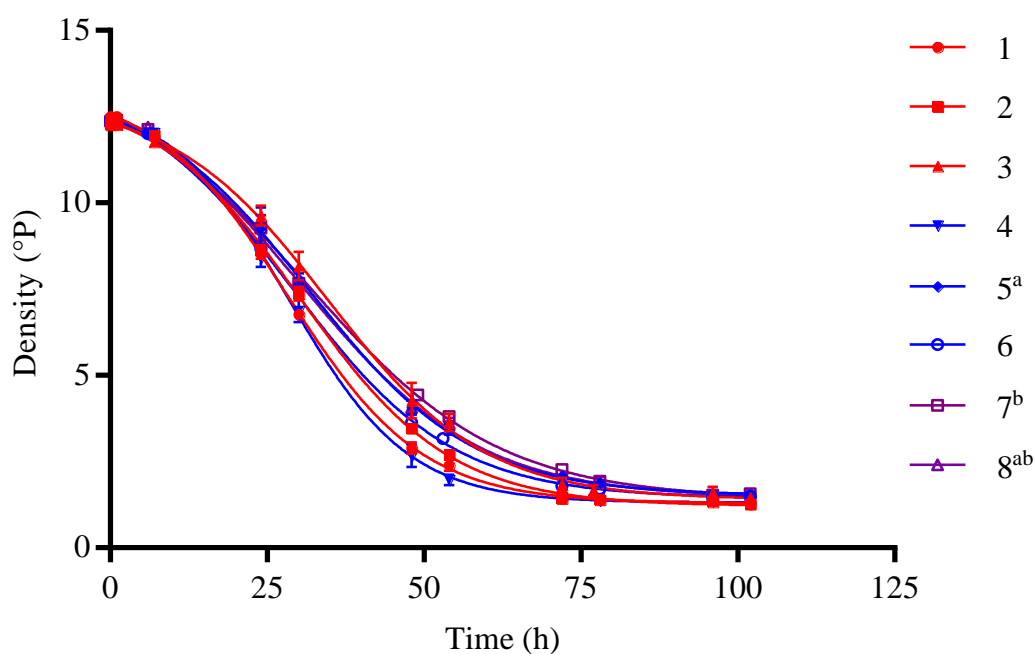
**Figure 7.2.** The density attenuation for eight serially re-pitched fermentations with yeast stored for 18 hours between fermentations. Each model represents three independent fermentations. The superscripts demonstrate the models that are identical by an F-Test.

Density attenuation trends for the fermentations using yeast stored for 42 hours between fermentations were also monitored (Figure 7.3). Similar to the fermentations with 18 hour yeast storage treatment, these attenuation trends were significantly different from the other attenuation trends in the series with two exceptions. The exceptions were fermentations two and eight, and fermentations three, five, and seven (Figure 7.3). Between these fermentations, the density attenuation rates were not significantly different.

Finally, the attenuation rates for the longest yeast storage time, which was 66 hours, were analysed (Figure 7.4). Most of these attenuation trends were also significantly different from the other attenuation trends in the series with two exceptions. These exceptions were fermentations five and eight, fermentations seven and eight (Figure 7.4).

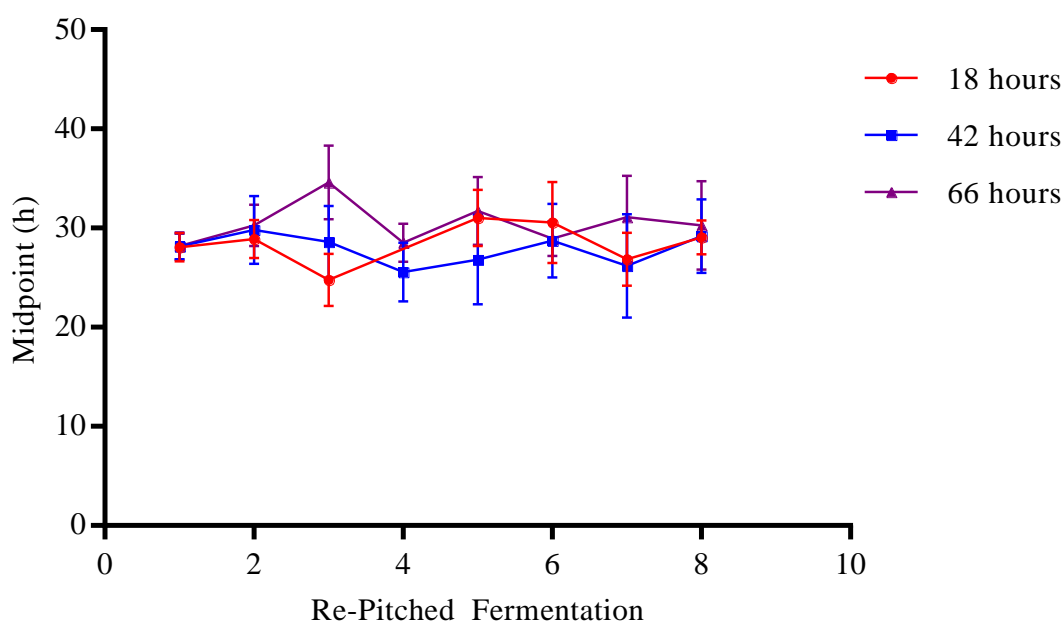


**Figure 7.3.** The density attenuation for eight serial re-pitched fermentations with yeast stored for 42 hours between fermentations. Each model represents three independent fermentations. The superscripts demonstrate the models that are identical by an F-Test.



**Figure 7.4.** The density attenuation for eight serial re-pitched fermentations with yeast stored for 66 hours between fermentations. Each model represents three independent fermentations. The superscripts demonstrate the models that are identical by an F-Test.

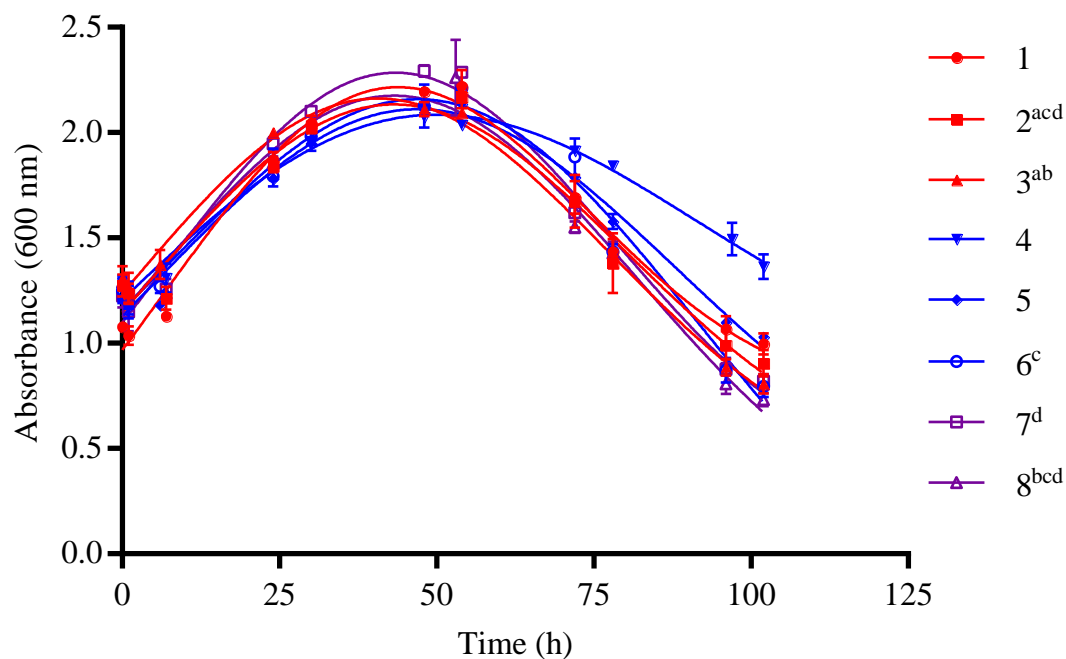
Linear regression was performed to determine if the midpoint of the fermentation was explained by the re-pitch number. No significant increase or decrease in midpoint (hour) was found that could be related to the crop number (Figure 7.5).



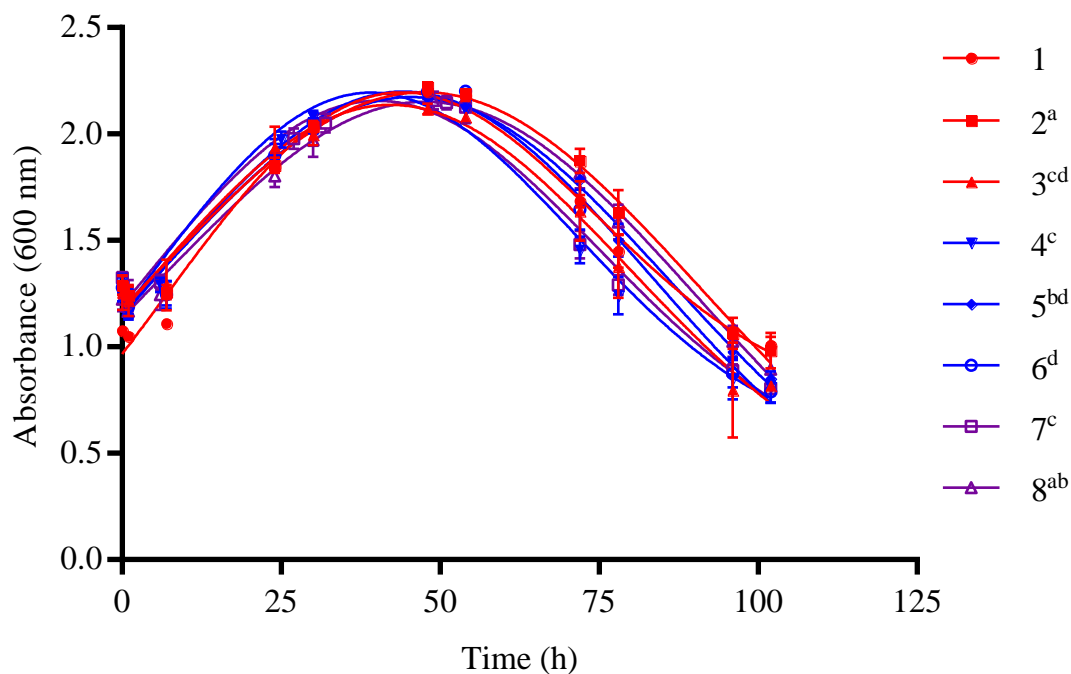
**Figure 7.5.** The midpoint of the fermentation compared to the re-pitched number. No significant linear regressions were found for re-pitched fermentations with 18, 42, or 66 hour storage time between fermentations.

Finally, the other characteristic of the physical fermentation monitored, was the yeast in suspension trends. In general, the absorbance measurements increased until approximately hour 50 of the fermentation. Once the yeast in suspension reached maximum growth, absorbance declined throughout the rest of fermentation.

In the re-pitched series where yeast was stored for 18 hours between fermentations, there were four instances where two or three fermentations were not significantly different (Figure 7.6). The re-pitched series where the yeast was stored for 42 hours between fermentations, also had four instances where two or three fermentations were not significantly different (Figure 7.7).

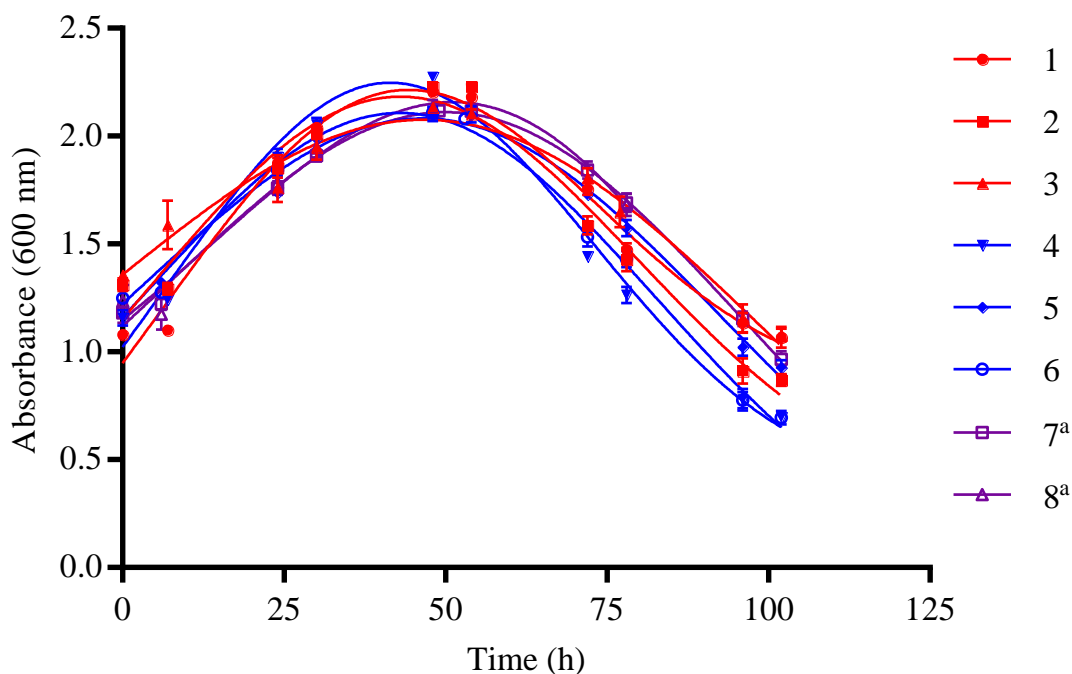


**Figure 7.6.** The yeast in suspension trends measured by absorbance (600nm) between eight serially re-pitched fermentations when the yeast was stored for 18 hours between fermentations. Each model represents three independent fermentations. The superscripts demonstrate the models that are identical by an F-Test.



**Figure 7.7.** The yeast in suspension trends measured by absorbance (600nm) between eight serially re-pitched fermentation when the yeast was stored for 42 hours between fermentations. Each model represents three independent fermentations. The superscripts demonstrate the models that are identical by an F-Test.

The re-pitched fermentation series with the yeast stored for 66 hours between fermentations had the lowest number of instances where the yeast in suspension trends were statistically the same. Only yeast in suspension trends for fermentations 7 and 8 were statistically the same model (Figure 7.8).

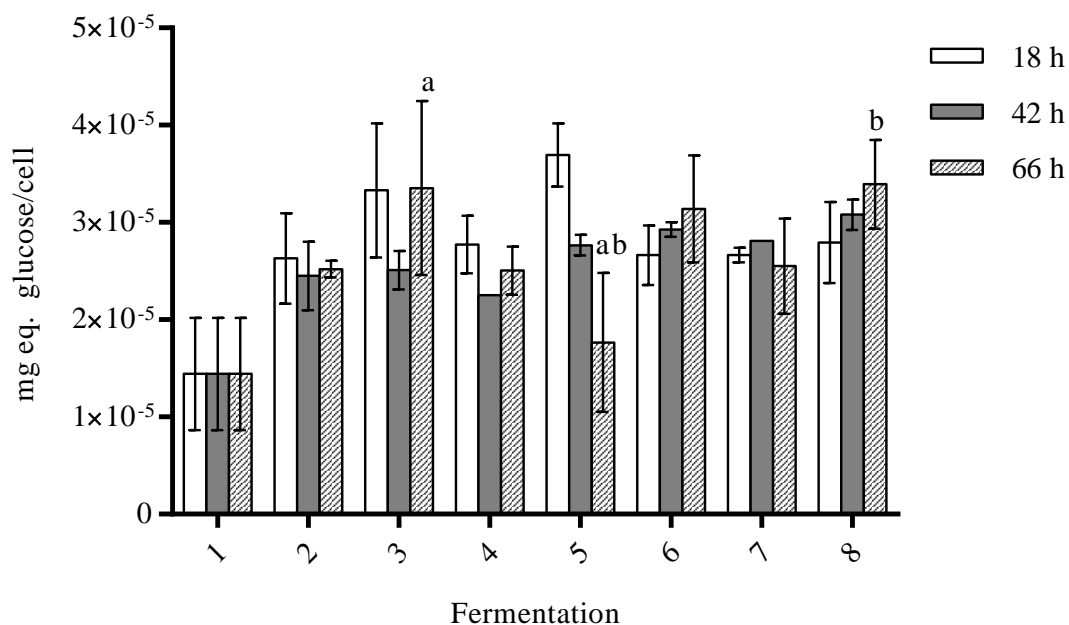


**Figure 7.8.** The yeast in suspension trends measured by absorbance (600nm) between eight serial re-pitched fermentations when the yeast was stored for 66 hours between fermentations. Each model represents three independent fermentations. The superscripts demonstrate the models that are identical by an F-Test.

### 7.3.2 Yeast Characteristics

The glycogen levels in the cell at the beginning of each fermentation were quantified (Figure 7.9). The results showed that glycogen levels were the lowest at the beginning of the first fermentation at  $1.44 \pm 0.58 \times 10^{-6}$  mg equivalent (eq.) glucose/cell. These cells were freshly propagated. At the beginning of the re-pitched fermentations, the glycogen levels were significantly higher in many cases than freshly propagated yeast (Table 7.1). This was determined by a two-way ANOVA test followed by Tukey's multiple comparisons. The yeast cultures that were stored for 18 and 42 hours showed no significant changes in glycogen levels at the beginning of fermentation between all re-pitched fermentations (fermentations 2-8) (Figure 7.9). When yeast was stored for 66 hours between re-pitched fermentations, the glycogen levels in the yeast culture between fermentations three and five, and fermentations five and eight were determined to be statistically significantly different.





**Figure 7.9.** Glycogen (in mg eq. glucose/cell) levels in pitched yeast prior to each serial re-pitched fermentation. Each bar represents 2-3 replicates and the error bars represent the standard deviation. The glycogen levels that are statistically significantly different between re-pitched fermentations for each treatment are marked with a letter, except for the first fermentation.

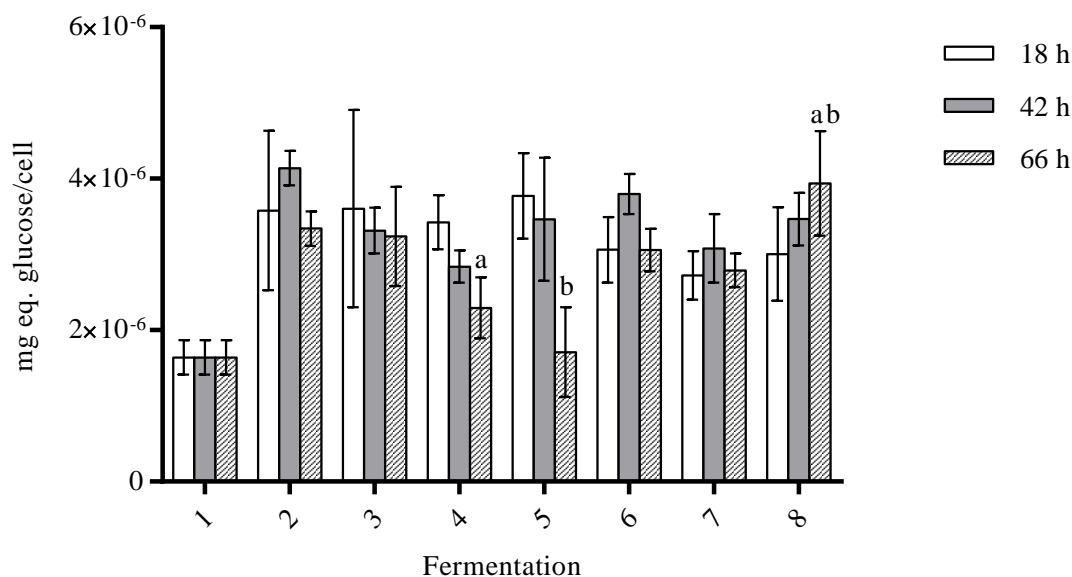
**Table 7.1.** Tukey's multiple comparisons test showing statistically significantly different glycogen levels at the start of fermentation between the first fermentation with freshly propagated yeast and the stated re-pitched fermentation (2-8).

Storage Time (h)	Significance between levels of glycogen at the start of the re-pitched Fermentation compared to fermentation one <sup>a</sup>						
	2	3	4	5	6	7	8
18	ns	***	*	****	*	*	*
42	ns	ns	ns	ns	**	*	**
66	ns	***	ns	ns	**	ns	***

<sup>a</sup> ns =  $p > 0.05$ , \*  $p \leq 0.05$ , \*\* =  $\leq 0.01$ , \*\*\* =  $p \leq 0.001$ , \*\*\*\* =  $p \leq 0.0001$

The trehalose levels in the cell at the beginning of each fermentation were also quantified. Freshly propagated yeast had the lowest trehalose concentrations at  $1.64 \pm 0.23 \times 10^{-6}$  mg eq. glucose/cell. The yeast cultures that were stored for 18 or 42 hours showed no statistically significant changes in trehalose levels at the beginning of fermentation between the re-pitched fermentations (fermentations 2-8) (Figure 7.10). When the yeast was stored for 66 hours between fermentations, trehalose levels in the yeast culture between fermentations 4 and 8, and fermentations 5 and 8 were statistically significantly different (Figure 7.10). This determined by a two-way ANOVA test followed by Tukey's multiple comparisons. As previously observed with cellular glycogen levels from the first fermentation (Table 7.1), the trehalose levels were

significantly lower in freshly propagated yeast than many cultures sampled from many re-pitched fermentations (Table 7.2).



**Figure 7.10.** Trehalose (mg eq. glucose/cell) levels in pitched yeast prior to each serial re-pitched fermentation. Each bar represents two to three replicates and the error bars represent the standard deviation. The trehalose levels that are statistically significant between re-pitched fermentations for each treatment are marked with a letter, except for the first fermentation.

**Table 7.2.** Tukey's multiple comparisons test showing statistically significant trehalose levels at the start of fermentation between the first fermentation with freshly propagated yeast and the stated re-pitched fermentation (2-8).

Storage Time (h)	Re-pitched Fermentation compared to Fermentation one <sup>a</sup>						
	2	3	4	5	6	7	8
18	**	**	**	***	ns	ns	ns
42	***	*	ns	*	***	ns	*
66	*	*	ns	ns	ns	ns	***

<sup>a</sup> ns =  $p > 0.05$ , \*  $p \leq 0.05$ , \*\* =  $\leq 0.01$ , \*\*\* =  $p \leq 0.001$ , \*\*\*\* =  $p \leq 0.0001$

Additionally, the yeast crops' viabilities were quantified at the start of fermentations; all yeast crops remained about 96 % viable cells. A two-way ANOVA showed that there was no significant difference between the yeast storage time and the crops' viabilities.

**Table 7.3.** Yeast viabilities at the start of each fermentation

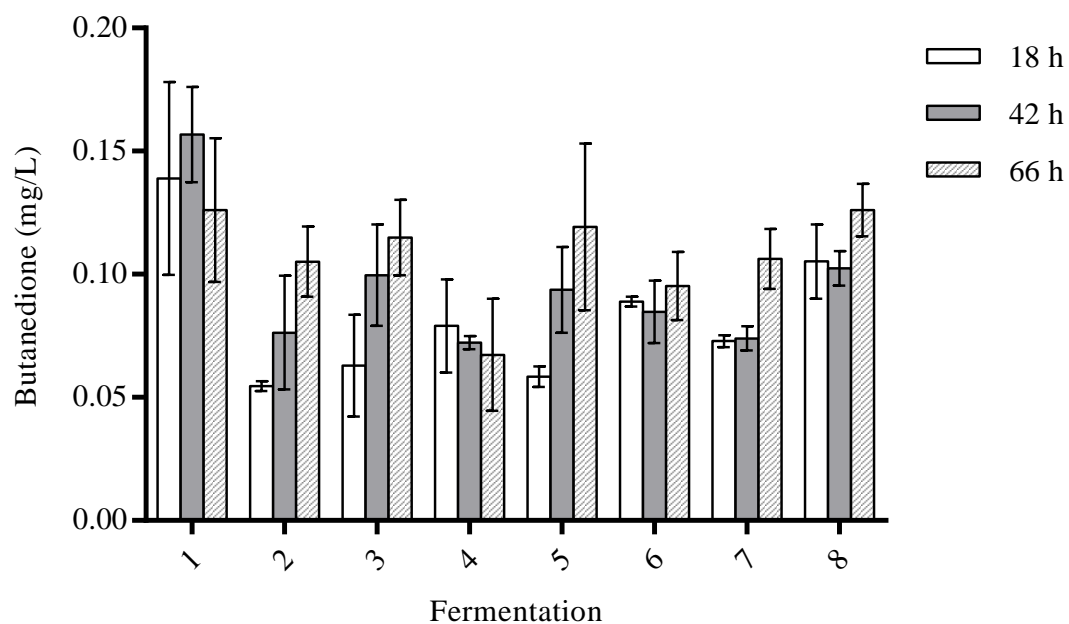
Fermentation	18 hours			42 hours			66 hours		
	Average	SD	n	Average	SD	n	Average	SD	n
1	99.20	0.18	3	99.20	0.18	3	99.20	0.18	3
2	97.93	0.50	3	97.63	0.65	3	97.76	0.24	3
3	98.42	0.51	3	98.13	0.35	3	98.38	0.25	3
4	98.33	0.20	3	97.74	0.27	3	96.20	0.59	3
5	97.40	0.69	3	98.77	0.26	3	98.85	0.19	3
6	98.39	0.22	3	98.22	0.52	3	98.05	0.41	3
7	98.57	0.22	3	98.48	0.59	3	98.80	0.22	3
8	98.95	0.26	3	98.81	0.11	3	98.37	0.32	3

\*SD = standard deviation; n = sample size

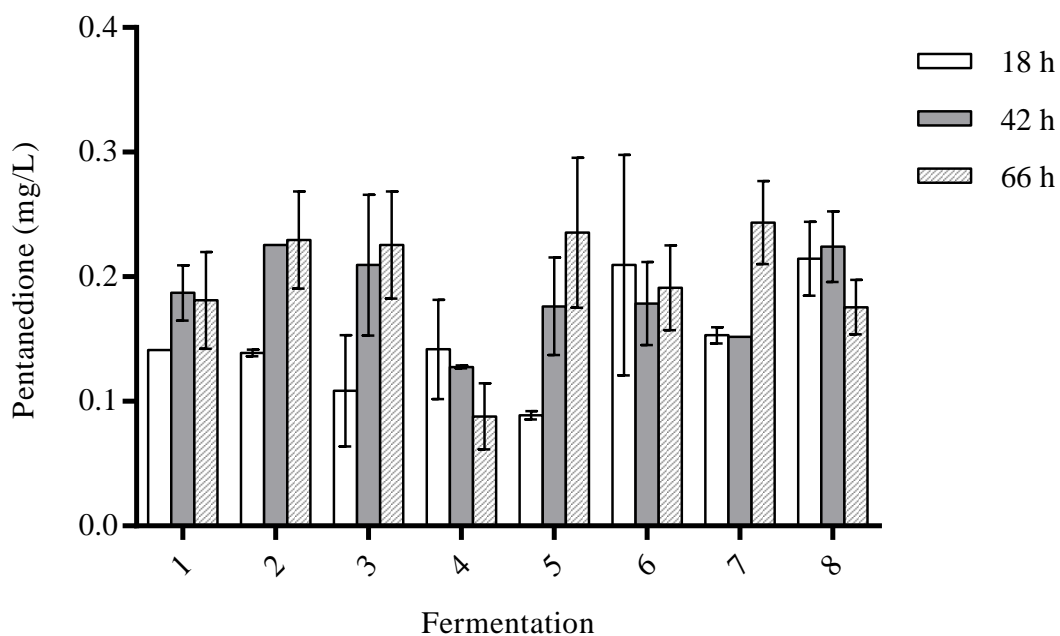
### 7.3.3 Flavour Results

Levels of vicinal diketones, higher alcohols, esters, and carbonyl compounds were analysed using samples from hour 102 of the fermentation. The concentrations of 2,3-butanedione at the end of fermentations varied from 0.04 – 0.17 mg/L (Figure 7.11) and levels of 2,3-pentanedione varied from 0.06 – 0.27 mg/L (Figure 7.12).

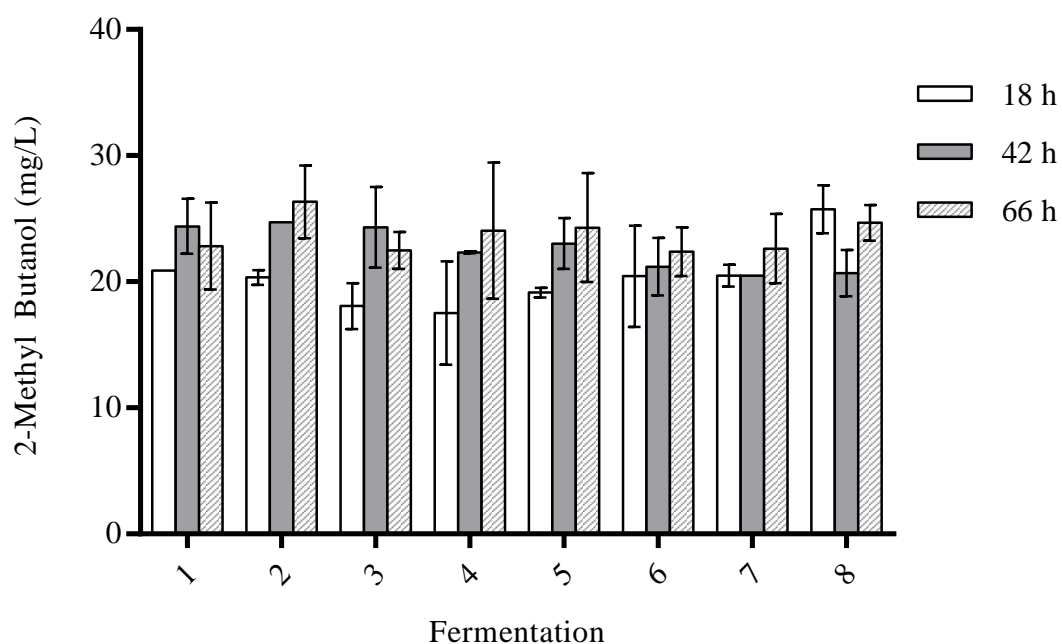
The higher alcohols were found in much larger quantities than the vicinal diketones. Concentrations of 2-methyl butanol ranged from 13.0 – 28.3 mg/L (Figure 7.13). For 3-methyl butanol, concentrations were higher than 2-methyl butanol at 50.3 – 87.6 mg/L (Figure 7.14). This compound also had the largest range in concentrations between all fermentations. The concentrations of propan-1-ol were the lowest of the four higher alcohols analysed and those concentrations varied 1.5 – 6.5 mg/L (Figure 7.15). The last higher alcohol analysed was isobutanol and those levels ranged from 8.9 – 35.8 mg/L (Figure 7.16).



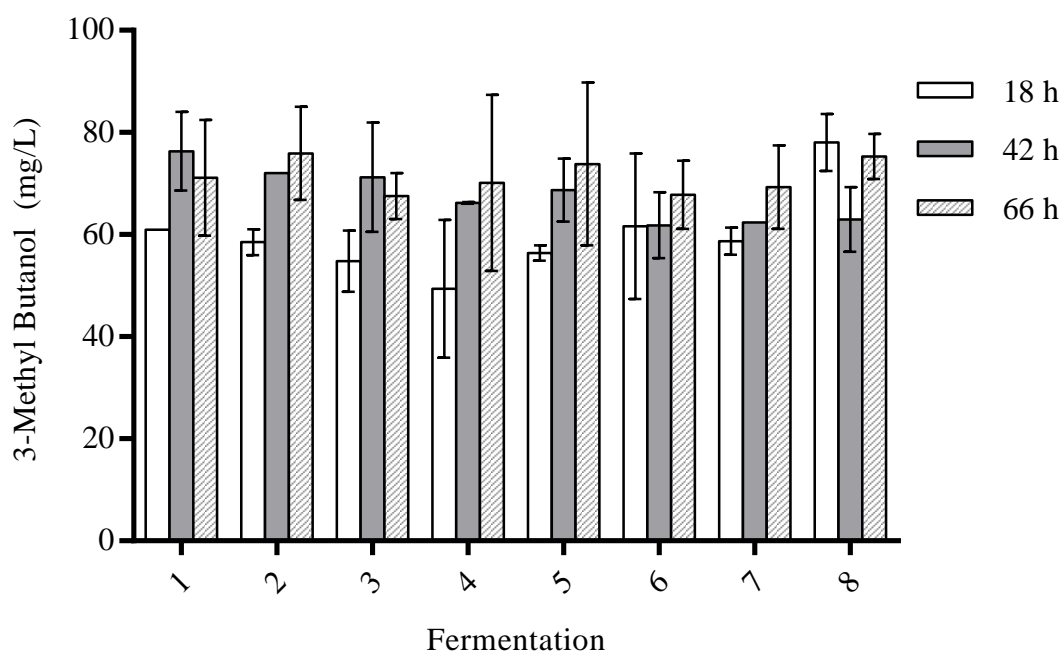
**Figure 7.11.** Levels of 2,3-butanedione post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



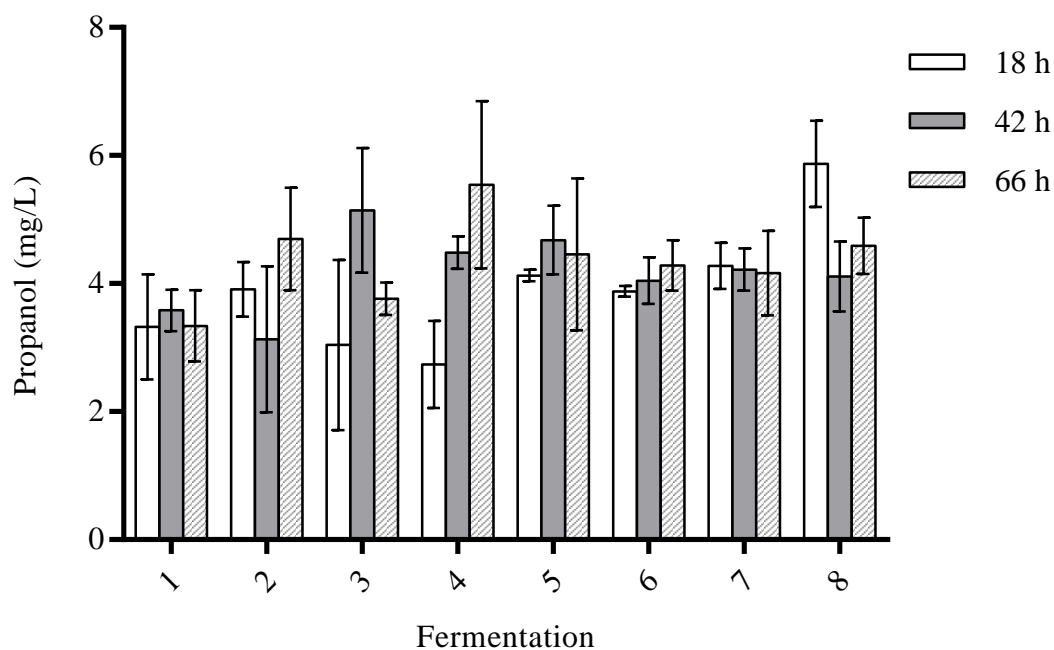
**Figure 7.12.** Levels of 2,3-pentanedione post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



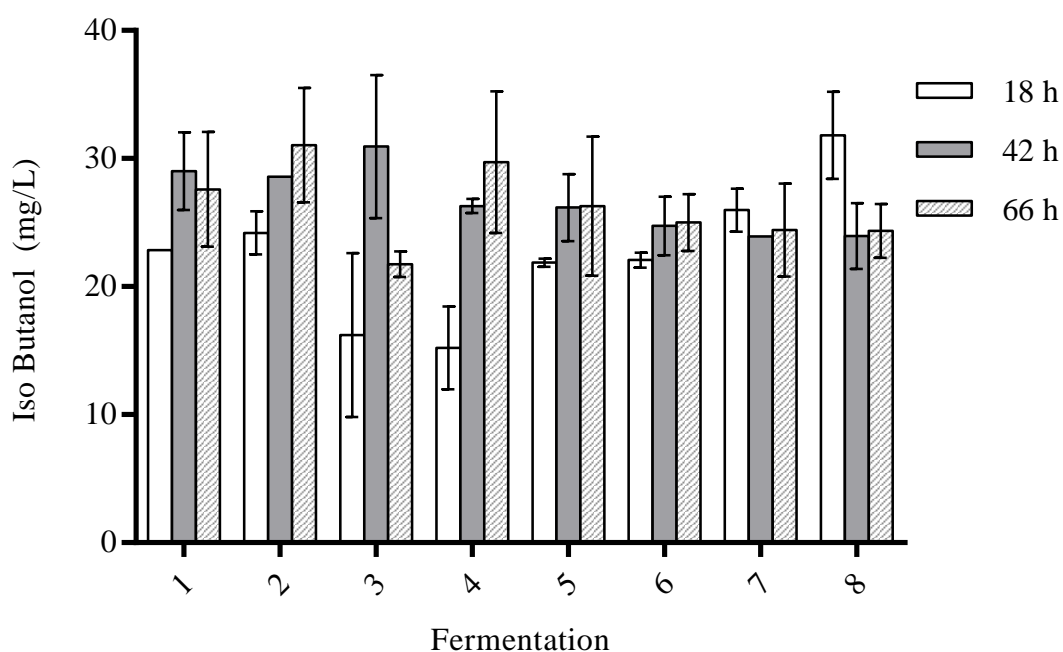
**Figure 7.13.** Levels of 2-methyl butanol post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



**Figure 7.14.** Levels of 3-methyl butanol post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



**Figure 7.15.** Levels of propan-1-ol post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



**Figure 7.16.** Levels of isobutanol post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).

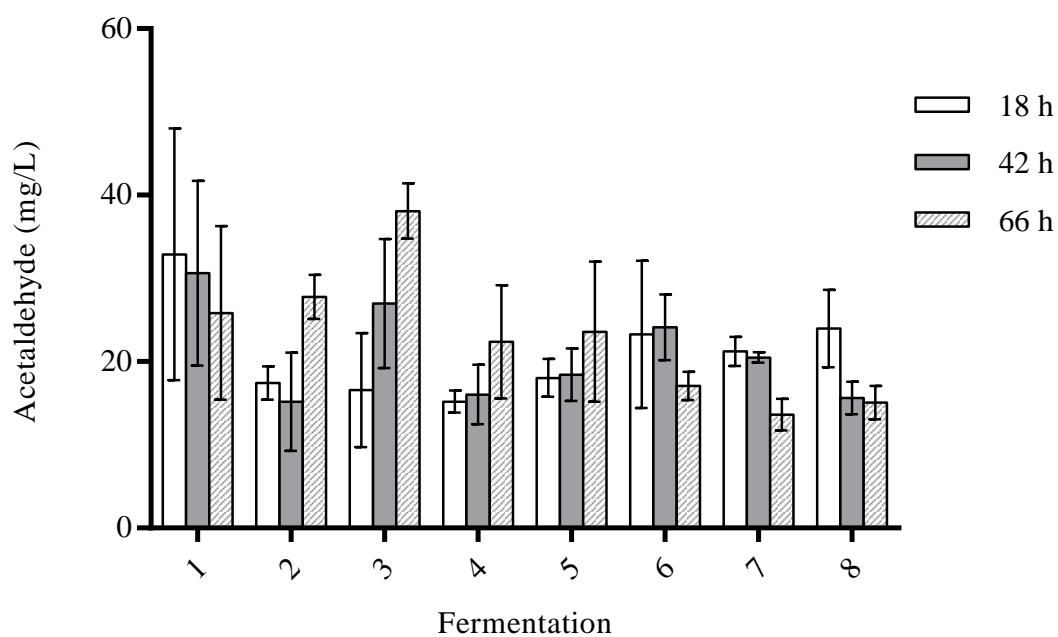
A two-way ANOVA was performed on the flavour data and to determine if the storage treatment or the re-pitched number had an overall effect on the flavours that were produced in the fermentations. The storage treatment had a significant effect on 2,3-butanedione, 2,3-pentanedione, 2-methyl butanol, and 3-methyl butanol. These flavour compounds were significantly or at least parametrically higher at the end of fermentation when stored for 66 hours when compared to 18 hour storage time with a few exceptions. This was not the case for levels of 2,3-butanedione at the end of fermentation four. For levels of 2,3-pentanedione, fermentations four and eight were exceptions. The exception with this trend observed for levels of 2-methyl butanol and 3-methyl butanol was fermentation eight (Table 7.4).

**Table 7.4.** Significance report from a two-way ANOVA that showed whether storage time, crop number, or both had a significant effect on the various flavour compounds analysed. Fermentation one was eliminated for this analysis because the results from the first fermentation did not undergo the storage time treatment.

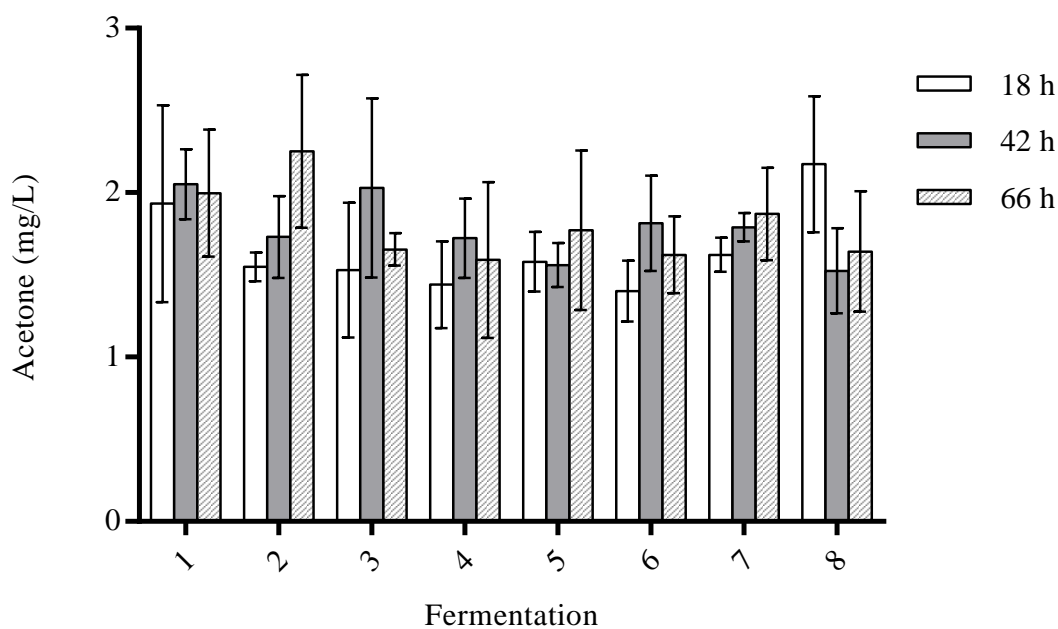
Flavour Compound	Level of significance <sup>a</sup>		
	Storage Time	Crop Number	Interaction
<b>Carbonyl</b>			
Acetaldehyde	ns	**	****
Acetone	ns	ns	ns
<b>Esters</b>			
Ethyl Acetate	ns	**	**
Ethyl Hexanoate	*	ns	ns
Isoamyl acetate	ns	***	**
Ethyl butyrate	ns	***	**
<b>Higher Alcohols</b>			
Isobutanol	**	ns	***
Propan-1-ol	ns	ns	***
2 methyl butanol	***	ns	ns
3 methyl butanol	***	ns	ns
<b>VDKS</b>			
2,3-Butanedione	****	***	*
2,3-Pentanedione	**	**	**

<sup>a</sup> ns = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$

The carbonyl compounds quantified were acetaldehyde and acetone. The concentrations of acetaldehyde were between 8.7 – 43.6 mg/L (Figure 7.17). For acetone, the concentrations ranged from 1.1 – 2.4 mg/L (Figure 7.18). There were no observable trends between the levels of acetaldehyde or acetone and the re-pitched fermentation or storage treatment.



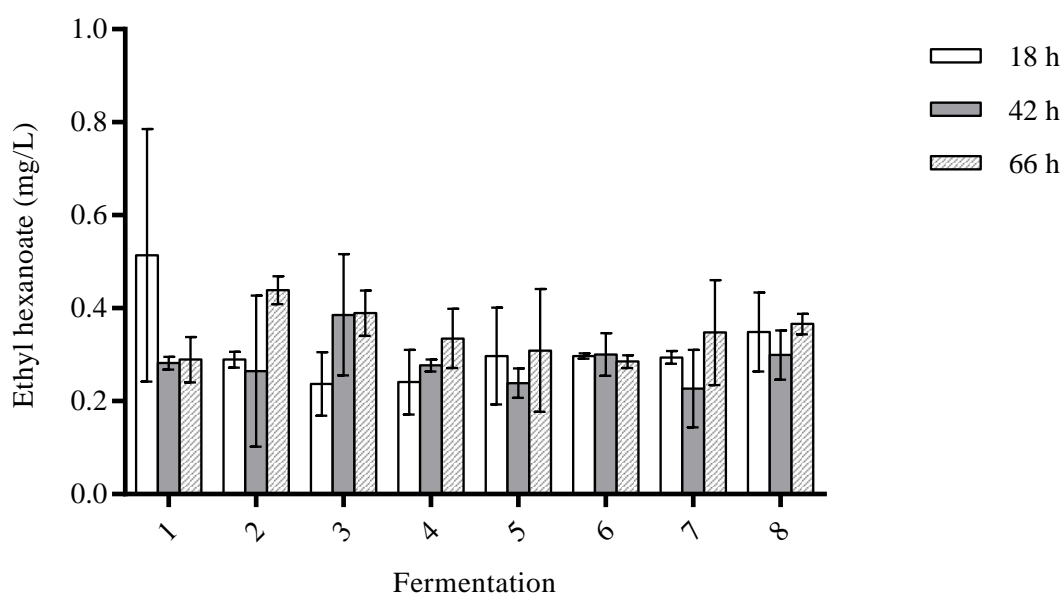
**Figure 7.17.** Levels of acetaldehyde post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



**Figure 7.18.** Levels of acetone post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).

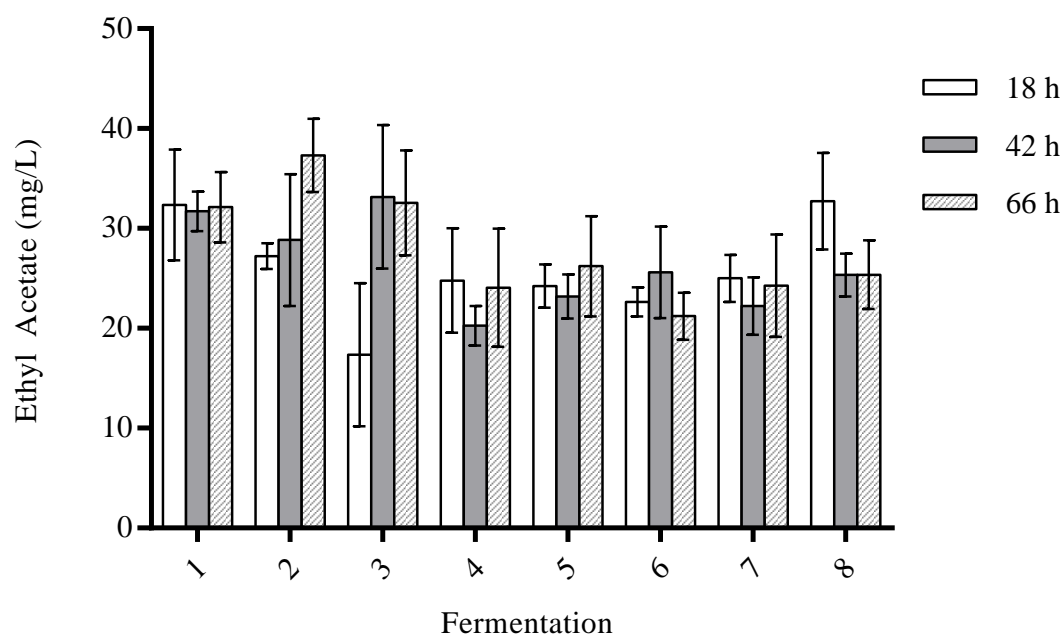


The esters investigated were ethyl acetate, ethyl hexanoate, ethyl butyrate, and isoamyl acetate. Ethyl hexanoate levels ranged from 0.2 to 0.7 mg/L (Figure 7.19). Ethyl acetate was present at the end of fermentation in the highest concentrations from 9.3 to 38.9 mg/L (Figure 7.20). Ethyl butyrate was present in the smallest concentrations from 0.1 to 0.2 mg/L (Figure 7.21). Lastly, levels of isoamyl acetate were determined to be between 0.8 to 4.2 mg/L (Figure 7.22). There were no observable trends between the esters and either the storage treatment, except for ethyl hexanoate. In the re-pitched fermentations, except for fermentation six, the levels of ethyl hexanoate were higher for the 66 hour yeast storage treatment compared to the 18 hour treatment (Figure 7.19). This observed difference was found to be significant (Table 7.3).

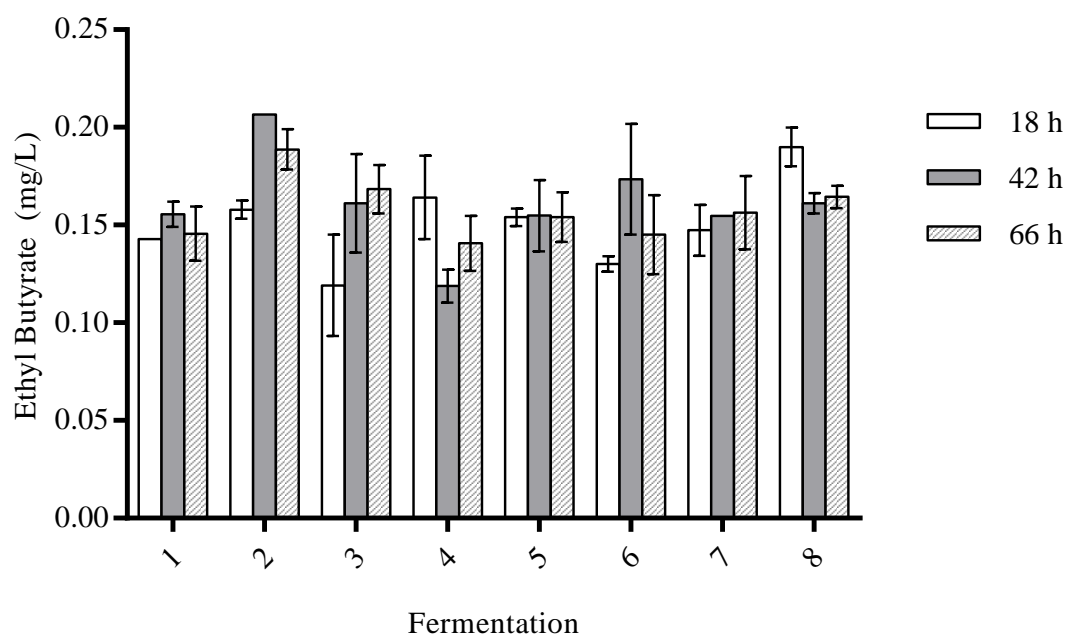


**Figure 7.19.** Levels of ethyl hexanoate post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).

There were variations with the levels of esters present at the end of fermentation and the re-pitched number (Table 7.3). The levels of ethyl acetate were dynamic between fermentation one to fermentation three for all storage treatments (Figure 7.20). The levels of ethyl acetate were constant from fermentation four to fermentation eight, with the exception of fermentation eight and the 18 hour yeast storage treatment. The levels of ethyl butyrate at the end of fermentation were inconsistent with the re-pitched number and storage treatment (Figure 7.21).

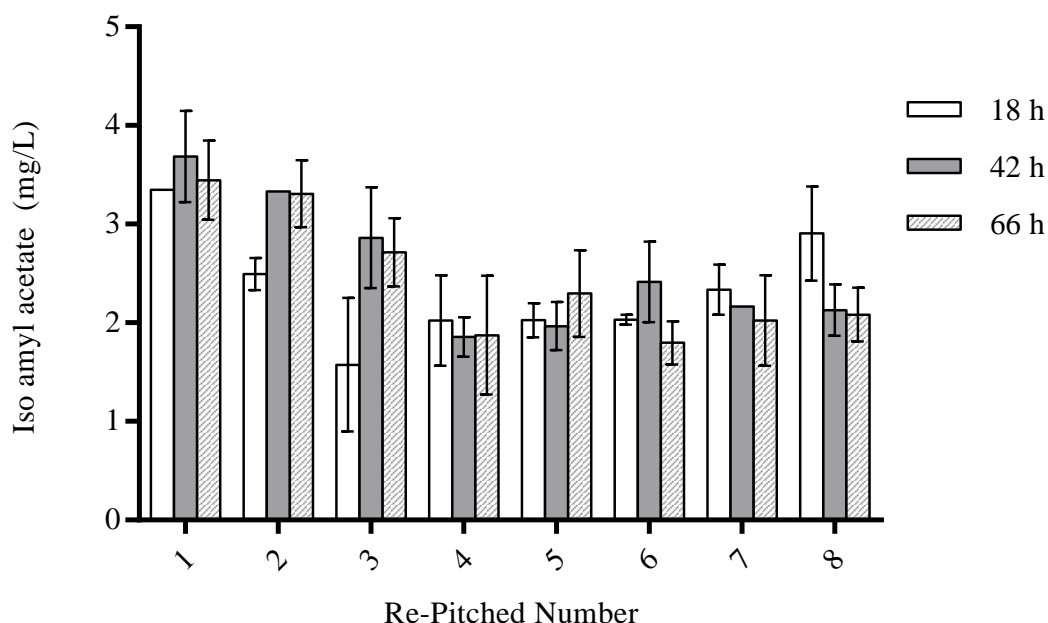


**Figure 7.20.** Levels of ethyl acetate post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).



**Figure 7.21.** Levels of ethyl butyrate post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).

The levels of isoamyl acetate were highest in the first fermentation with freshly propagated yeast (Figure 7.22). A decline in isoamyl acetate at the end of fermentation was observed between re-pitched fermentations until fermentation four. After fermentation four, the levels of isoamyl acetate remained constant for all treatments with the exception of fermentation eight for the 18 hour yeast storage treatment.



**Figure 7.22.** Levels of isoamyl acetate post primary fermentation in re-pitched yeast that had different storage treatments between fermentations (18 h, 42 h, or 66 h).

## 7.4 Discussion

### 7.4.1 Physical Fermentation

During the re-pitched fermentation series with yeast stored for 18 hours between fermentations, fermentation four took the longest time to final density (Figure 7.2). Correspondingly the absorbance for fermentation four remained higher at the end of fermentation compared to all others. Unfortunately, this result correlated with an equipment malfunction during the storage time prior to this fermentation. Normally, yeast was stored at 4 °C between fermentations. However, during the 18 hour storage of the yeast culture prior to fermentation four, the fridge broke down and the temperature of the yeast slurry reached 21 °C by the time the yeast culture was retrieved for preparation for the next fermentation. The yeast cultures from the other two treatments were fermenting at the time and were unaffected by the fridge malfunction. It is highly likely that the slower fermentation was related to the sporadic higher storage temperature. However, because the replicates were not performed at different times, all triplicates were exposed to this. For this reason, we cannot be certain that the aberrant

fermentation profile was by cause of the high storage temperature and not the re-pitched number with this storage time. Somani and colleagues (2012) showed that when yeast were stored at higher temperatures for 24 hours, the glycogen levels were much lower than those in yeast that were stored at 4 °C and 10 °C. This was not observed in this experiment (Figure 7.9). However, it is suggested that this discrepancy could be a result of different storage times. The storage temperature for the yeast cultures started at 4 °C prior to the fridge malfunction. It was likely that there was a slow increase in temperature until the yeast storage temperature reached 21 °C prior to re-pitching, and was not held at 21 °C for the entire 18 hours. Taking these considerations into account, it was likely the aberrant fermentation profile was due to the modification in yeast storage temperature.

Similar to the density attenuation trends between re-pitched fermentations from the chapters that investigated industrial lager and ale yeast (Chapters 4 and 5), linear regression trends for crop number compared to the midpoint of the fermentation were not significant (Figure 7.5). This showed that the speed of the fermentation was unchanged with respect to the increasing crop number. Furthering this observation, within each fermentation series for all treatments, there were multiple instances when two or three density attenuation models were not significantly different (Figure 7.1, 7.2, and 7.3). In one instance, the density attenuation models between fermentation two and fermentation eight for the series with 42 hour yeast storage were not significantly different. The attenuation rate at the end of the fermentation series was identical to an attenuation rate at the beginning. This demonstrated that fermentative ability stayed conserved throughout this series and this did not pose any reason to discard the yeast culture after the eighth fermentation. This was also the case for the fermentation series with yeast stored for 18 hours where fermentations one and seven had density attenuation rates that were not significantly different. When the yeast was stored for 66 hours between fermentations, the lowest number of instances where two density attenuation curves were not significantly different when compared to the other two storage treatments were observed. These instances took place at the end of the re-pitched fermentation cycle. It is suggested that the culture takes a longer time to adapt to the re-pitched environment when the storage time is longer. Furthering this observation, visual inspection of density attenuation trends showed that the re-pitched fermentations for 18 hour yeast storage were more consistent than the 66 hour yeast storage density attenuations models.

The yeast in suspension trends also suggested that the re-pitched fermentation series with the yeast stored for 66 hours exhibited larger variations between fermentations. Again, the 66 hour yeast storage treatment had fewer instances where the trends for the 8 serial re-pitched fermentations were not significantly different. This time, the trends observed were for yeast in suspension, and not density attenuation. There was only one instance where two trends were not significantly different and this was between fermentations 7 and 8. With the shorter yeast storage treatments for the re-pitched fermentations, there were four instances in each treatment where two or three yeast in suspension trends were identical. This signified that the yeast growth during the first half of fermentation, and the yeast flocculation nearing the end of fermentation, was the same between those fermentations.

Yeast storage conditions are low in nutrients and high in ethanol (Gibson *et al.*, 2007). It is hypothesized that with longer exposure to these conditions, the yeast's ability to transition to fermentation decreased. This was supported with the observation that re-pitched fermentation cycle with the longest yeast storage time, had more variation in both the yeast in suspension trends and density attenuations. Even though this variation was observed in yeast in suspension and density attenuation trends, the % viable cells in the crops remained the same between each yeast storage time treatments.

#### **7.4.2 Yeast Storage Carbohydrates**

Yeast cells accumulate glycogen and trehalose in aerobic or anaerobic environments when nitrogen or carbon is limited (Lillie and Pringle, 1980; Parrou *et al.*, 1999; Thomsson *et al.*, 2005). During yeast propagation, yeast have access to excess nutrients, therefore, glycogen accumulation would be unexpected. Additionally, if yeasts are pitched into a medium of high gravity, all other nutrients need to be adjusted to higher levels to accommodate the higher gravity content (Gibson, 2011). There are multiple promoters for the synthesis of glycogen in the yeast cell, so if any nutrients are limiting, then glycogen accumulation would take place. Any of these limiting nutrients could be a nitrogen, carbon, sulphur, or phosphorus source (François and Parrou, 2001). The data collected in this study aligned with this literature. Glycogen levels in freshly propagated yeast at the beginning of the first fermentation were significantly lower than several glycogen levels at the start of re-pitched fermentations (Table 7.1). This trend was also found with trehalose levels in the cell at the start of fermentation (Table 7.2). This also demonstrated that glycogen and trehalose levels were higher in re-pitched yeast than freshly propagated yeast. Again, this was expected because nutrients are

limited towards the end of fermentation. Therefore, glycogen and trehalose levels would accumulate during this time.

During storage between fermentations, the yeast cells were exposed to an environment where nutrients were exhausted. During storage, energy for any yeast metabolism that takes place comes from intracellular carbohydrate reserves. Somani and colleagues (2012) conducted a study where propagated yeast was stored at different temperatures and the glycogen and trehalose levels were assessed over a time period. They showed that regardless of temperature the cells glycogen and trehalose levels decreased during storage (Somani *et al.*, 2012). In the serial re-pitching cycle, yeast rely on glycogen and trehalose accumulation at the end of fermentation to provide energy during the storage period and at the beginning of fermentation following re-pitching. The glycogen and trehalose levels were higher than freshly propagated yeast in the re-pitched yeast after the storage time and this showed that more glycogen and trehalose accumulated during the end of fermentation than was utilized during the storage period 18, 42, or 66 h.

When the yeast in the current study was stored for 18 hours and 42 hours, there was no significant change in glycogen and trehalose levels observed in the cell at the start of fermentation between all re-pitched fermentations. It is suggested that with 18 hours or 42 hours of yeast storage between re-pitched fermentations, that the yeast culture could recover and maintain consistent glycogen and trehalose levels. With 66 hour yeast storage between fermentations, there were instances where the glycogen and trehalose between re-pitched fermentations were significantly different. This was interestingly the re-pitched fermentation series where the density attenuation rates had the largest variation between fermentations compared to the other two storage treatments. This showed that with 66 hour yeast storage between fermentations, the yeast cells were also unable to maintain consistent glycogen and trehalose levels.

#### **7.4.3 Flavour Data**

The storage time had an overall significant effect on the levels of 3-methyl butanol, 2-methyl butanol, isobutanol, 2,3-butanedione, 2,3-pentanedione, and ethyl hexanoate. That effect was an overall increase in levels of the specific flavour compounds when the yeast was stored longer between fermentations. These levels of higher alcohols and vicinal diketones were below or approximately the level of the flavour thresholds for all fermentations and treatments (Pires and Brányik, 2015). The levels of ethyl hexanoate were present (0.2 – 0.7 mg/L) either at or above the 0.23 mg/L

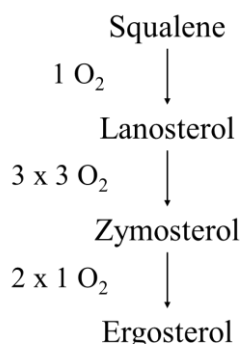
flavour threshold (Pires and Brányik, 2015). All other flavour compounds were below or approximately the same value as the flavour threshold. Therefore, those differences in flavour compounds observed would likely be undetected in the beer. These results imply that ethyl hexanoate would be the only quality concern in the final product.

Even though storing the yeast between 18 to 66 hours would likely play a minor role in the quality of the beer, the significant results suggest the storage time influenced yeast metabolic activity. In this study, longer storage times were associated with increased levels of 2,3-butanedione, 2,3-pentanedione, isobutanol, 2-methyl butanol, and 3-methyl butanol. Higher alcohol and vicinal diketone productions are both related to the amino acid metabolism (Pires and Brányik, 2015). Higher alcohol production is increased with increased absorption of amino acids from the wort (Pires *et al.*, 2014). Levels of 2,3-butanedione, however, are decreased with an increased absorption of amino acids, specifically valine (Krogerus and Gibson, 2013). It is proposed that with longer storage times, the absorption of amino acids from the wort increases, and this may lead to increased levels of higher alcohols. Theoretically, this would also lead to lower levels of 2,3-butanedione. If this hypothesis were true, then the increased 2,3-butanedione levels were possibly longer storage times affecting the cells ability to reduce 2,3-butanedione to 2,3-butanediol. This speculation would need further study to be validated.

## Chapter 8 – The Effect of Wort Oxygenation Levels on Extended Serial Re-Pitching

### 8.1 Introduction

Yeasts function as facultative anaerobes during fermentation, however, the cells utilize oxygen during the lag period for biosynthesis of unsaturated fatty acids and sterols (Section 1.2.4 and Section 1.3.5). Shortly after pitching the yeast, the Crabtree Effect occurs. This effect states that when high concentrations of sugars are present, yeasts' aerobic respiration pathway is repressed, and fermentation takes place (Kirsop, 1974; Barford and Hall, 1979). Therefore, the beginning of fermentation is the only time when sterol and unsaturated fatty acid synthesis occurs. Ergosterol is the predominant sterol found in the yeast cell, and the biosynthesis of this compound requires a large quantity of molecular oxygen to be utilised (Figure 8.1) (Rosenfeld *et al.*, 2003). Unsaturated fatty acids also require oxygen for synthesis. The major unsaturated fatty acids found in yeast cells are palmitoleic acid and oleic acid (Rosenfeld and Beauvoit, 2003).



**Figure 8.1.** Simplified schematic of the biosynthesis of ergosterol in the cell and oxygen requirement for each step (Rosenfeld and Beauvoit, 2003).

Gradually anaerobic fermentation takes place in the fermenting medium as the yeast depletes the dissolved oxygen (Gibson *et al.*, 2007) and some dissolved oxygen goes towards wort oxidation (O'Rourke, 2002). Normally, a 12 °P wort requires 8 – 12 mg/L dissolved oxygen (DO) at the start of fermentation to enable a complete fermentation, with sufficient yeast growth (Gibson *et al.*, 2008b). A recent study demonstrated that fermentation reached its final density quicker with 12 mg/L of DO in the wort than with lower DO levels (7 and 10 mg/L) (Kucharczyk and Tuszyński, 2017). The same study demonstrated that with higher wort DO levels, the yeast growth was greater during fermentation. These authors also found that the highest DO treatment resulted in significantly higher levels of propan-1-ol and amyl alcohols, and



significantly lower levels of acetaldehyde (Kucharczyk and Tuszyński, 2017). Additionally, lower concentrations of esters have been observed in other studies with increased wort oxygenation levels (Verstrepen *et al.*, 2003; Bühligen *et al.*, 2013). The observation from Kucharczyk and Tuszyński (2017) with the levels of acetaldehyde was similar to another study (Maemura *et al.* 1998). That study showed that levels of acetaldehyde were highest at the end of fermentation when yeast that was propagated anaerobically compared to aerobically propagated yeast (Maemura *et al.*, 1998).

When wort oxygen concentration is insufficient for yeast metabolism, the primary impact is lower yeast growth during fermentation (Kirsop, 1974). In the same piece of work, Kirsop (1974) proposed that yeast can be classified (Table 8.1) based on the discovery that different yeast strains have an optimal DO requirement for fermentation. For example, one strain, NCYC 1236, can grow on all malt wort with an initial DO concentration as low as 2 ppm (Kirsop, 1974). Another study built on this work and demonstrated that when some strains were exposed to excess oxygen, the effect was detrimental to yeast growth during fermentation (Jakobsen and Thorne, 1980).

**Table 8.1.** Proposed concepts of different classes or groups of yeast strains by their oxygen requirements (Kirsop, 1974).

Class	Yeast's oxygen requirements
01	Yeast is satisfied by half air saturated wort
02	Yeast is satisfied by air saturated wort
03	Yeast is satisfied by oxygen saturated wort
04	Yeast is unsatisfied by oxygen saturated wort

The work presented investigated the accumulated effects of how ‘low’ concentrations of DO (3 mg/L), ‘normal’ concentrations of DO (8 mg/L) and ‘high’ concentrations of DO (16 mg/L) in the wort impacted the physical fermentation, flavour compounds produced, and yeast lipid concentrations over the course of three serial re-pitched fermentations. It was hypothesized that if the wort was under oxygenated, the yeast cells would be unable to synthesize the required sterols and unsaturated fatty acids. Furthermore, stuck fermentations and aberrant flavour profiles would be observed, and ultimately, serial re-pitching would be unsustainable. It was also

hypothesized that 16 mg/L of wort oxygenation would not affect the sterol and unsaturated fatty acid levels in the yeast cells, but would affect the flavour profiles in the beer produced when compared to worts with 8 mg/L DO.

## 8.2 Experimental Design

An appropriate quantity of yeast (strain SMA) was propagated in YEPD media to pitch 2.7 L of wort (Section 2.3.1) at a concentration of  $1.5 \times 10^7$  viable cells/mL. The yeast slurry was washed (Section 2.3.1) and counted (Section 2.5.2) prior to pitching. Prepared wort (Section 2.2.3) was thawed for 3 days at 4 °C and autoclaved (121 °C, 15 psi) to ensure sterility. The wort was adjusted to a density of 12.6 °P using sterile water.

The wort was adjusted to three levels of DO:  $3.1 \pm 0.5$  mg/L,  $8.1 \pm 0.2$  mg/L, and  $16.2 \text{ mg/L} \pm 0.3 \text{ mg/L}$ . The saturation of DO levels in water has been extensively researched and charts are available that shows saturation of DO in water at different temperatures (Kolev, 2012). However, the presence of solids in the wort (i.e. sugars, proteins, polyphenols) decreases the solubility of oxygen in the liquid media. Ward and Dageforde (1966) demonstrated that the solubility of oxygen in 12 °P wort is approximately 75 – 80 % the solubility of oxygen in water. In fact, in 12 °P wort at 10 °C, the solubility of oxygen in wort is approximately 9.5 mg/L (Ward and Dageforde, 1966). Similarly, a later study found the solubility of oxygen in 12 °P wort was 9.3 mg/L at 10 °C (Krauss, 1967). The solubility of oxygen in wort would only decrease as the gravity increases. Given this, the DO measurements from the probe used for the current study reached 20 mg/L, which would be theoretically impossible. The probe used was a calibrated LDO oxygen probe (Hach Ltd., Manchester, UK) This optical probe has previously been reported to be utilized specifically for brewing wort (Fitzgerald and Nimptsch, 2016). Additionally, there have been papers that reported higher levels of DO above the previously mentioned saturation levels of oxygen in wort (Cunningham and Stewart, 2000). Cunningham and Stewart (2000) completed a study with reported 12 ppm (approximately 12 mg/L) DO levels in 12 °P wort. Additionally, Kucharczyk and Tuszyński (2017) reported performing an experiment with 12 mg/L DO in 15.5 °P wort. In view of this controversy, the measurements in this experiment should be considered relative values.

To achieve the different initial DO treatments, the wort was then split into two aliquots. Sterile oxygen was bubbled into one aliquot of wort by spinning with a magnetic flea at approximately 3 psi for five minutes to achieve  $16.2 \text{ mg/L} \pm 0.3 \text{ mg/L}$  DO. A 20 mL sample was removed and placed in a 25 mL beaker to test the DO level

using a calibrated LDO oxygen probe. The remaining aliquot was placed under a vacuum pump and stirred with a magnetic flea for approximately 2 hours to lower the DO content of the wort below 1 mg/L. The DO concentration in the wort for this treatment was  $3.1 \pm 0.5$  mg/L of dissolved oxygen. The oxygen level was tested using the LDO probe after the wort was pitched and transferred into the fermenter. The order was performed because the DO of the wort increased slightly during the transfer. To achieve wort containing  $8.1 \pm 0.2$  mg/L of DO, the wort aliquots that contained 1 mg/L and 16 mg/L DO were blended.

Henry's law (Equation 8.1) states that at equilibrium, the amount of gas that was dissolved in liquid is proportional to the partial pressure in the same gas in the atmosphere above the liquid (Ward and Dageforde, 1966).

$$p_g = K \times N_g$$

(Equation 8.1)

The parameter  $p_g$  in Henry's law defines the partial pressure of the gas,  $K$  defines Henry's Law Coefficient, and  $N_g$  defines the mole fraction of the gas in the atmosphere. Conforming with Henry's law, all treatments would reach the same equilibrium of DO over time. However, during fermentation, this does not happen. Yeast produce  $\text{CO}_2$  during fermentation, and  $\text{CO}_2$  saturation of the wort normally takes place before the first 10 hours of fermentation (MacIntosh *et al.*, 2016). Additionally, the DO in the wort decreases over the course of the fermentation until it reaches approximately 0 mg/L DO by the end of the fermentation (Kucharczyk and Tuszyński, 2017). An initial experiment was performed to assess the risk of dissolved oxygen entering the medium at the beginning of fermentation. The beginning of fermentation was a concern because, the Crabtree effect within the yeast would not yet occur (Section 8.1). A 900 mL aliquot of wort with approximately 1.6 mg/L of DO was rested at room temperature for 80 min. This was the lowest DO concentration achievable. A sample was taken periodically from the surface of the fermenter and the DO concentration was measured and recorded.

For the preliminary experiment, the propagated yeast was pitched into 3 x 900 mL fermentations at  $1.5 \times 10^7$  cells/mL. The individual fermentations were sampled at hours 0, 1, 5, 7, 24, 30, 48, 54, 72, 78, 96, and 102 during the experiment and modelled according to the ASBC Yeast-14 protocol (Section 2.3.3).

At hour 102, a sample of the beer was taken for flavour analysis and the yeast was cropped. A 40 mL sample of beer was taken approximately 15 cm from the top of the fermenter for flavour analysis. The sample was placed in a 50-mL centrifuge tube and centrifuged (3000 x g, 5 min.). The supernatant was collected and stored at -60 °C in a 50 mL centrifuge tube until analysed for volatile flavour compounds (Section 2.9). To collect the cropped yeast, the beer was removed from the fermenter until approximately 30 mL beer remained. A sterile 25 mL glass pipette was utilized to collect yeast from the top of the yeast sediment. A 45 mL sample of yeast was transferred into a 50 mL centrifuge tube and the tube was centrifuged and the supernatant was discarded. A second 45 mL aliquot of the yeast sediment was added to the centrifuge tube. The tube was centrifuged and the supernatant discarded. A third 45 mL aliquot of yeast sediment was added. This time the yeast pellet was re-suspended using a vortex mixer.

A 3 mL sample of yeast was collected from the cropped yeast when it was cropped at hour 102. The yeast sample was “washed” three times as previously described. On the last wash, the pellet was re-suspended in 1 mL of sterile water and the yeast was transferred into a dry pre-weight vial. A 1mL aliquot of water was added two more times to the centrifuge tube to collect residual yeast and transferred into the pre-weighed vial. The tube was stored at -60 °C until all the samples were collected.

The tube that contained the rest of the cropped yeast was sealed with parafilm and stored at 4 °C for 41 hours until prepared for re-pitching (Section 2.5.2). The yeast was re-pitched for a total of three fermentations with the dedicated DO levels.

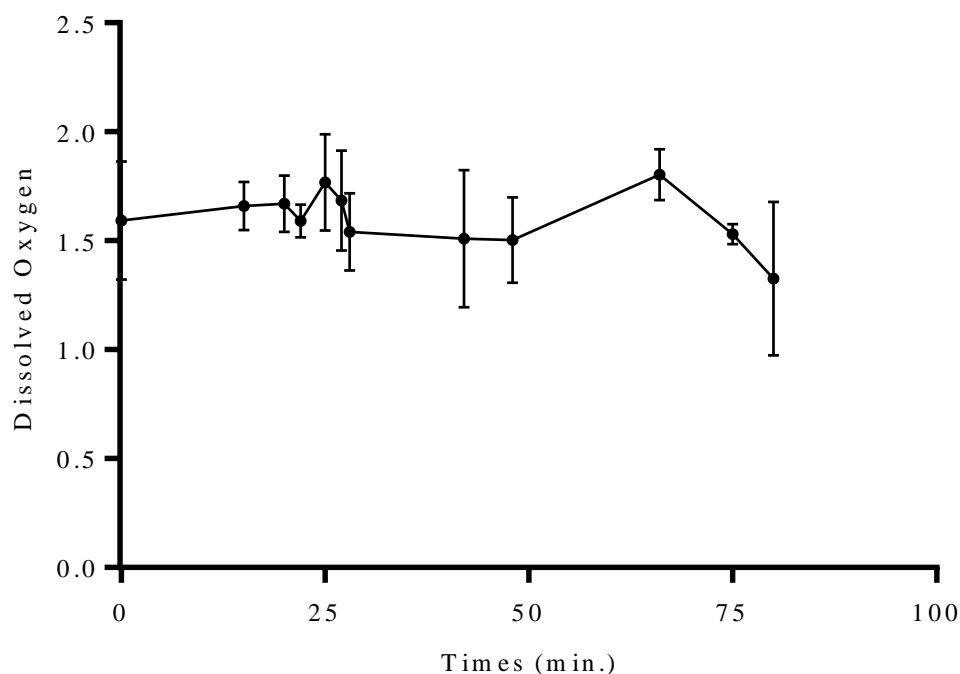
The frozen samples of yeast were freeze dried in a freeze dryer (Edwards Super Modulyo, Fisher Scientific, Loughborough, UK) for one week. After the samples were removed from the freeze dryer, the dried yeast and bottles without the lids were weighed. The lids were replaced and covered with Parafilm. The samples were stored at approximately 21 °C until analysed to determine the lipid profile (Section 2.14). All statistical tests with a  $p < 0.05$  were considered significant.

## 8.3 Results

### 8.3.1 *Dissolved Oxygen Equilibrium*

Before the fermentation experiment was performed, the DO level of non-inoculated wort was monitored. The level of DO started at  $1.6 \pm 0.3$  mg/L and after 80 minutes of resting at room temperature was  $1.3 \pm 0.4$  mg/L (Figure 8.2). For these

reasons, it was assumed that after the DO was measured immediately prior to inoculating the wort, the levels of DO only decreased. Therefore, the lowest treatment of 3 mg/L was never exposed to higher levels of DO.

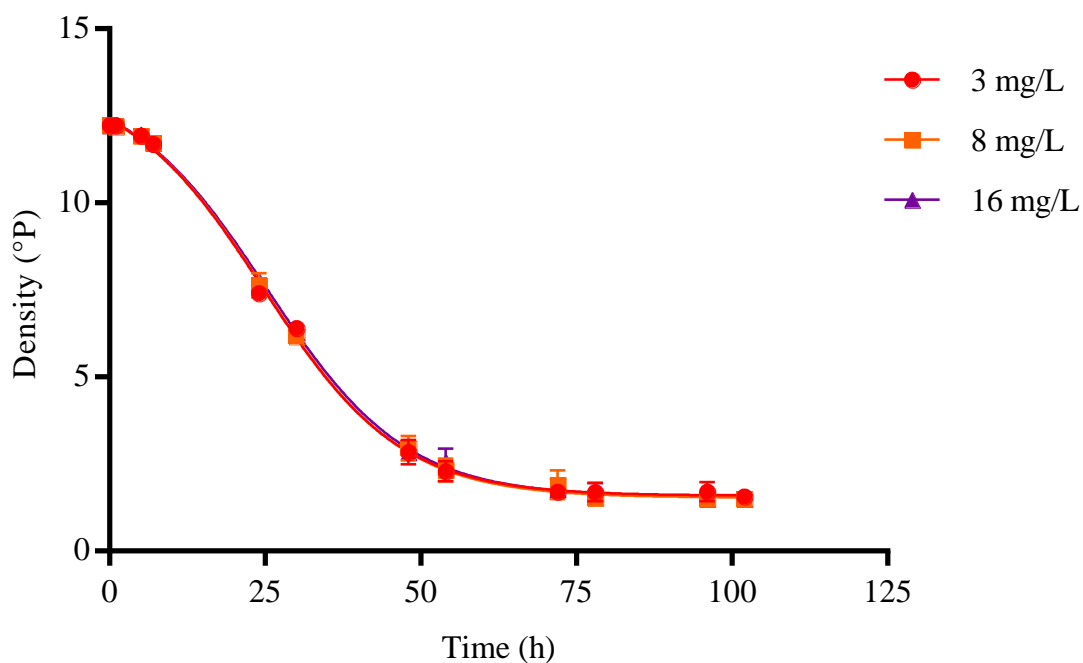


**Figure 8.2.** Levels of DO in the wort at the surface of a 900 mL fermenter not inoculated with yeast. Linear regression showed no significant linear trends as time increased with the DO level.

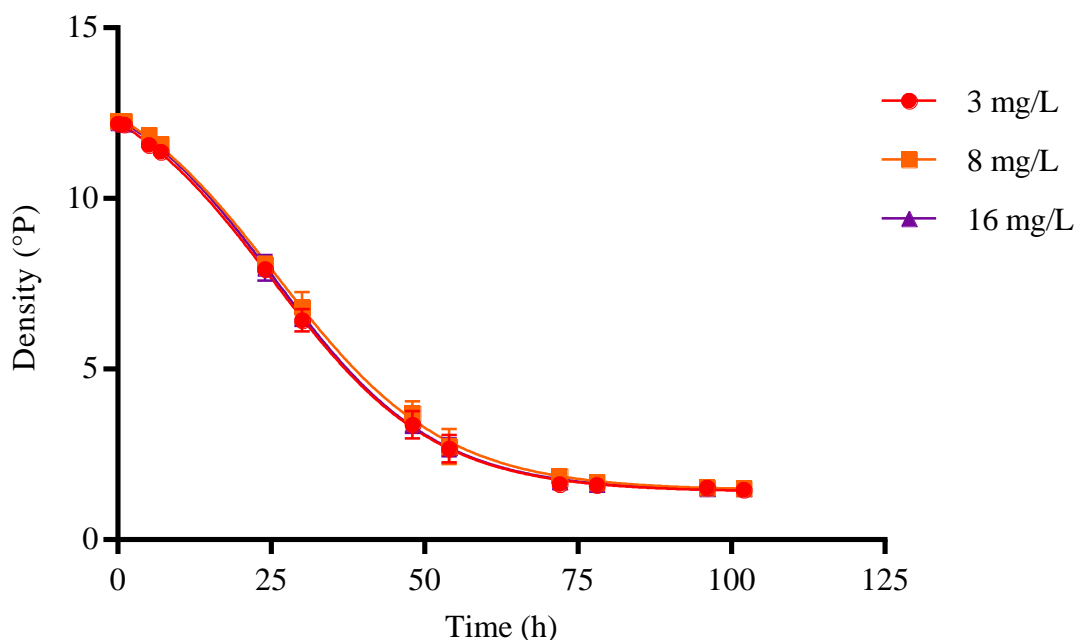
### 8.3.2 *Physical Fermentation*

The density attenuation models for all DO treatments were monitored throughout three consecutive fermentations. The density during fermentation for the three treatments started at 12.2 °P and all ended at  $1.5 \pm 0.1$  °P. The modelled attenuation for all treatments for this fermentation freshly propagated yeast were not significantly different (Figure 8.3).

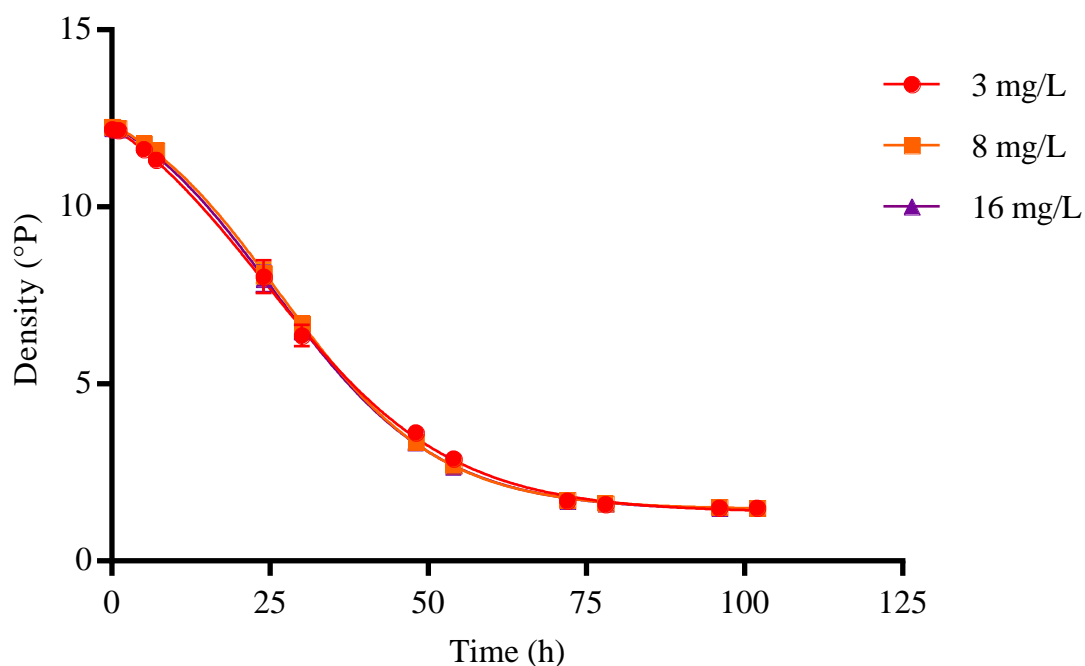
The density attenuation trends for the second fermentation and third fermentation also started at 12.2 °P and ended at  $1.5 \pm 0.05$  °P for all treatments. Within the second fermentation (Figure 8.4) and within the third fermentation (Figure 8.5), the attenuation models between all treatments were not significantly different.



**Figure 8.3.** Modelled density attenuation for the fermentation with freshly propagated yeast (fermentation one). The fermentations contained 3 mg/L oxygen (red circle), 8 mg/L (orange square) and 16 mg/L (purple triangle) DO at the start of fermentation. The three modelled nonlinear regressions were not significantly different by an F-Test.

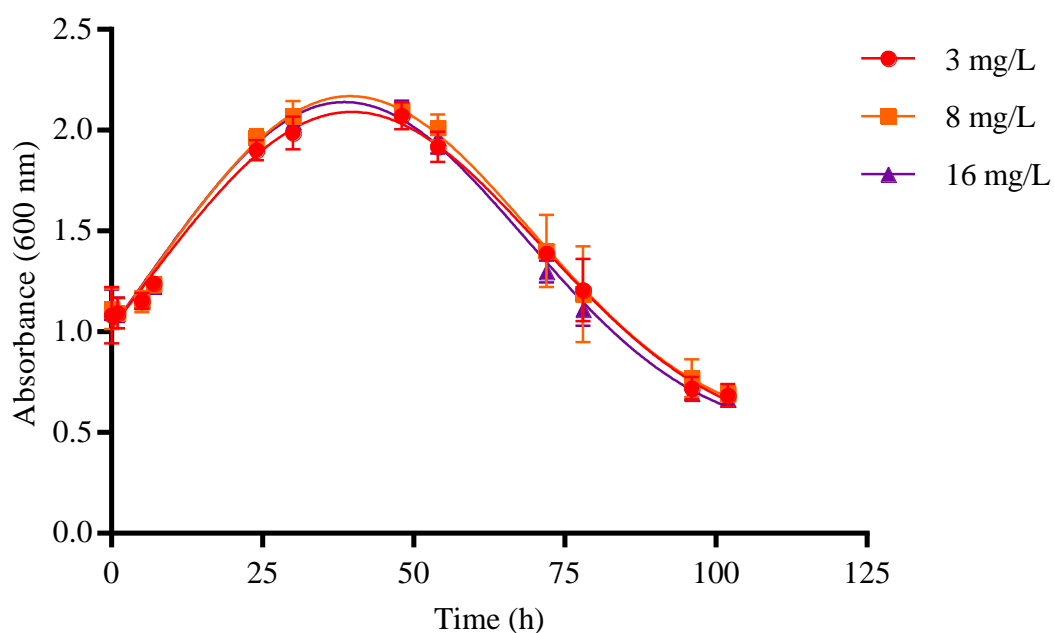


**Figure 8.4.** Modelled density attenuation for the fermentation with yeast re-pitched one time (fermentation two). The models represent fermentations that contain 3 mg/L (red circle), 8 mg/L (orange square) or 16 mg/L (purple triangle) DO concentrations. The three modelled nonlinear regressions were not significantly different by an F-Test.

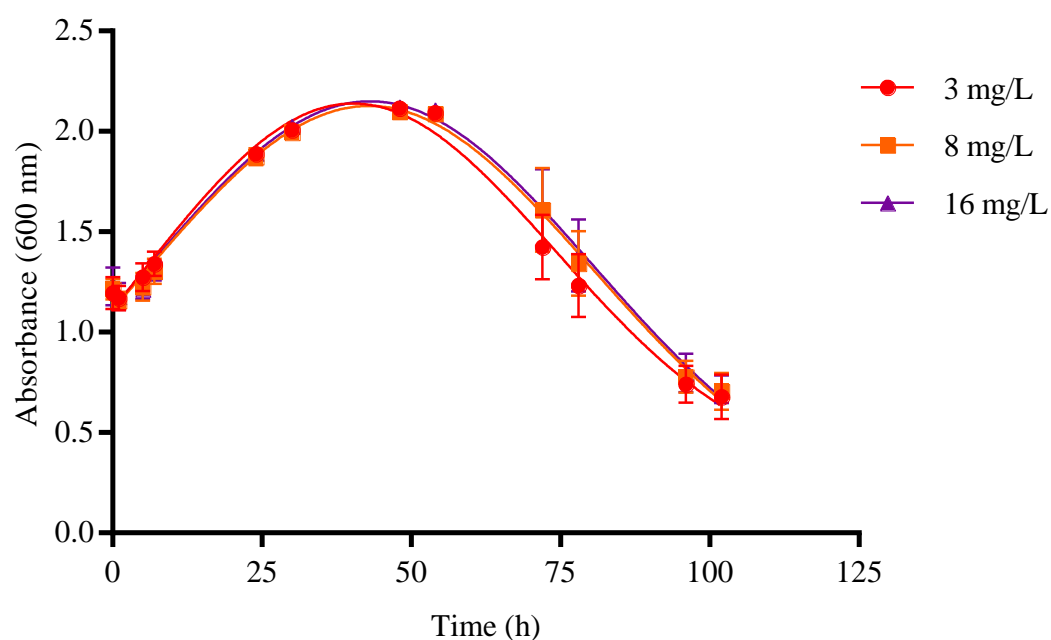


**Figure 8.5.** Modelled density attenuation for the fermentation with yeast re-pitched two times (fermentation three). The models represent fermentations that contain 3 mg/L oxygen (red circle), 8 mg/L (orange square) or 16 mg/L (purple triangle) initial DO concentrations. The three modelled nonlinear regressions were not significantly different by an F-Test.

The protocol followed that the absorbance measurements were taken at the same time as the density measurements throughout the fermentation. In the first fermentation, the maximum absorbance reached was between 2.087 and 2.165 for all treatments (Figure 8.6). In the second fermentation with re-pitched yeast, the maximum absorbance reached was between 2.127 and 2.150 for all treatments (Figure 8.7). Finally, the third fermentation with yeast re-pitched two times reached a maximum absorbance in the fermentation between 2.081 and 2.117 (Figure 8.8). This reflected the results from the density attenuation (Figure 8.3 – 8.5), appearing that the treatments had no effect on the yeast in suspension measurements within each fermentation.

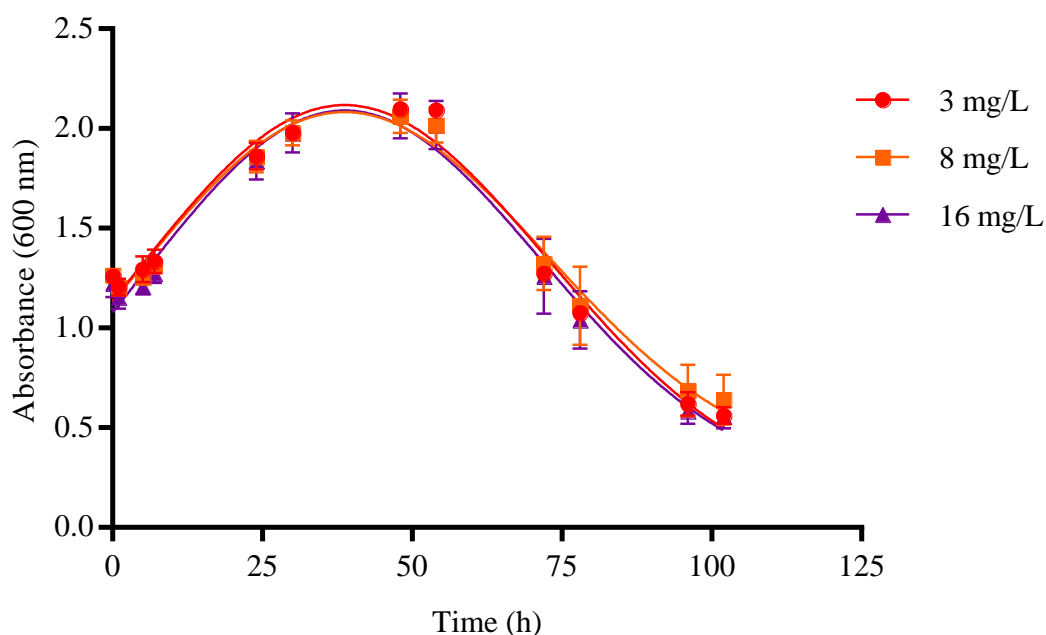


**Figure 8.6.** Modelled yeast in suspension trends with the fermentation with freshly propagated yeast. The models represent fermentations that contain 3 mg/L oxygen (red circle), 8 mg/L (orange square) or 16 mg/L (purple triangle) initial DO concentrations. The three modelled curves were not significant by an F-Test.



**Figure 8.7.** Modelled yeast in suspension trends with the fermentation with yeast re-pitched once (fermentation two). The models represent fermentations that contain 3 mg/L oxygen (red circle), 8 mg/L (orange square) or 16 mg/L (purple triangle) initial DO concentrations. The three modelled curves were not significant by an F-Test.





**Figure 8.8.** Modelled yeast in suspension trends with the fermentation with yeast re-pitched twice (fermentation three). The models represent fermentations that contain 3 mg/L oxygen (red circle), 8 mg/L (orange square) or 16 mg/L (purple triangle) initial DO concentrations. The three modelled curves were not significant as tested by an F-Test.

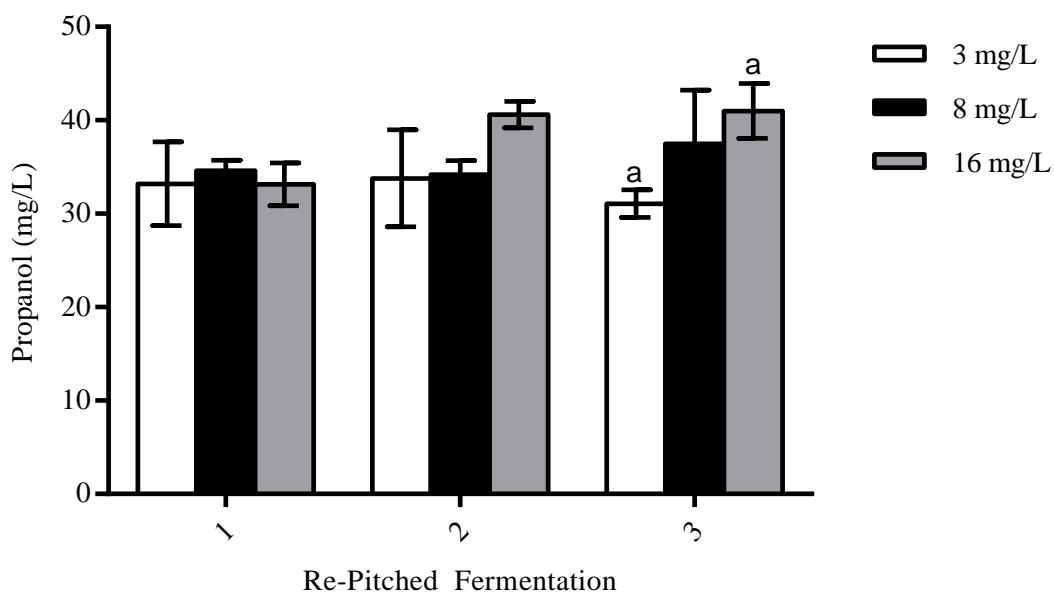
### 8.3.3 Beer Flavour Profile

The concentration of esters, carbonyl compounds, and vicinal diketones were analysed at the end of the primary fermentation (Table 8.2). With the exception of propan-1-ol and 2,3-butanedione, there were no significant differences found between the three treatments of DO during each fermentation for the all flavour compounds. Propan-1-ol showed a significant difference between the third re-pitching fermentation with 3 mg/L and 16 mg/L (Figure 8.9). For 2,3-butanedione, the concentrations determined between the treatments became significantly different during the third fermentation between 3 mg/L and 16 mg/L DO.

In addition to this, the fermentation number did show a significant effect on the levels of ethyl acetate, ethyl butyrate, ethyl hexanoate, 3-methyl butanol, and 2,3-butanedione. The concentrations of these esters were significantly lower at the end of the first fermentation than when compared to the second and third. The levels of 3-methyl butanol and 2,3-butanedione were significantly lower in the first fermentation when compared to the second and third. All other flavour compounds: acetaldehyde, acetone, propan-1-ol, isoamyl acetate, 2-methyl butanol, isobutanol, and 2,3-pentanedione overall had no significant differences between the crop number.

**Table 8.2.** Esters, carbonyl compounds, and higher alcohols analysed at hour 102 of each of the three re-pitched fermentations for each DO treatment (3 mg/L, 8 mg/L and 16 mg/L). The average of triplicate fermentations with the standard deviation (SD) is shown.

Flavour Compound	Ferm.	Average (mg/L beer) $\pm$ SD					
		3 mg/L DO		8 mg/L DO		16 mg/L DO	
Isoamyl Acetate	1	2.05	$\pm 0.23$	2.23	$\pm 0.19$	2.11	$\pm 0.35$
	2	2.30	$\pm 0.72$	2.56	$\pm 0.33$	2.66	$\pm 0.36$
	3	2.66	$\pm 0.15$	2.46	$\pm 0.24$	2.44	$\pm 0.28$
Ethyl hexanoate	1	0.26	$\pm 0.011$	0.22	$\pm 0.046$	0.22	$\pm 0.008$
	2	0.32	$\pm 0.062$	0.28	$\pm 0.02$	0.28	$\pm 0.036$
	3	0.32	$\pm 0.077$	0.30	$\pm 0.029$	0.28	$\pm 0.040$
Ethyl Acetate	1	20.10	$\pm 2.02$	21.65	$\pm 2.52$	22.94	$\pm 5.81$
	2	27.55	$\pm 7.32$	24.35	$\pm 2.35$	28.16	$\pm 4.22$
	3	28.87	$\pm 2.02$	26.43	$\pm 2.86$	27.44	$\pm 2.58$
Ethyl Butyrate	1	0.12	$\pm 0.0025$	0.12	$\pm 0.0023$	0.12	$\pm 0.0058$
	2	0.14	$\pm 0.032$	0.14	$\pm 0.0085$	0.16	$\pm 0.0060$
	3	0.16	$\pm 0.023$	0.14	$\pm 0.0068$	0.14	$\pm 0.0025$
Propan-1-ol	1	33.21	$\pm 4.48$	34.61	$\pm 1.14$	33.16	$\pm 2.29$
	2	33.79	$\pm 5.20$	34.18	$\pm 1.52$	40.60	$\pm 1.42$
	3	31.08	$\pm 1.49$	37.49	$\pm 5.74$	41.00	$\pm 2.95$
Isobutanol	1	25.96	$\pm 1.74$	27.01	$\pm 1.81$	25.75	$\pm 2.49$
	2	22.27	$\pm 5.29$	22.94	$\pm 1.96$	25.17	$\pm 0.067$
	3	19.81	$\pm 1.35$	22.79	$\pm 1.74$	24.84	$\pm 2.19$
2-Methyl Butanol	1	22.56	$\pm 1.19$	23.99	$\pm 2.71$	22.82	$\pm 2.44$
	2	20.94	$\pm 5.31$	20.09	$\pm 4.40$	23.44	$\pm 0.66$
	3	20.87	$\pm 2.01$	21.39	$\pm 1.82$	23.24	$\pm 2.39$
3-Methyl Butanol	1	69.86	$\pm 3.01$	74.64	$\pm 6.80$	72.25	$\pm 7.56$
	2	56.37	$\pm 15.06$	55.89	$\pm 14.43$	65.02	$\pm 0.88$
	3	57.02	$\pm 6.79$	60.66	$\pm 6.69$	67.02	$\pm 9.06$
Acetone	1	1.29	$\pm 0.23$	1.35	$\pm 0.048$	1.40	$\pm 0.11$
	2	1.50	$\pm 0.23$	1.37	$\pm 0.078$	1.36	$\pm 0.15$
	3	1.29	$\pm 0.068$	1.51	$\pm 0.22$	1.53	$\pm 0.11$
Acetaldehyde	1	13.81	$\pm 5.56$	13.49	$\pm 0.87$	12.76	$\pm 2.85$
	2	13.79	$\pm 3.70$	11.46	$\pm 2.57$	13.68	$\pm 3.29$
	3	16.00	$\pm 3.22$	15.90	$\pm 4.90$	15.56	$\pm 2.71$
2,3-Butanedione	1	0.14	$\pm 0.033$	0.12	$\pm 0.002$	0.14	$\pm 0.018$
	2	0.09	$\pm 0.020$	0.08	$\pm 0.028$	0.09	$\pm 0.010$
	3	0.14	$\pm 0.009$	0.09	$\pm 0.018$	0.10	$\pm 0.008$
2,3-Pentanedione	1	0.22	$\pm 0.058$	0.18	$\pm 0.014$	0.20	$\pm 0.014$
	2	0.17	$\pm 0.017$	0.17	$\pm 0.021$	0.20	$\pm 0.030$
	3	0.22	$\pm 0.036$	0.19	$\pm 0.022$	0.20	$\pm 0.010$



**Figure 8.9.** Levels of propan-1-ol in the three re-pitched fermentations with varying levels of DO (3 mg/L, 8 mg/L, and 16 mg/L). The significant difference between treatments is marked with an “a”.

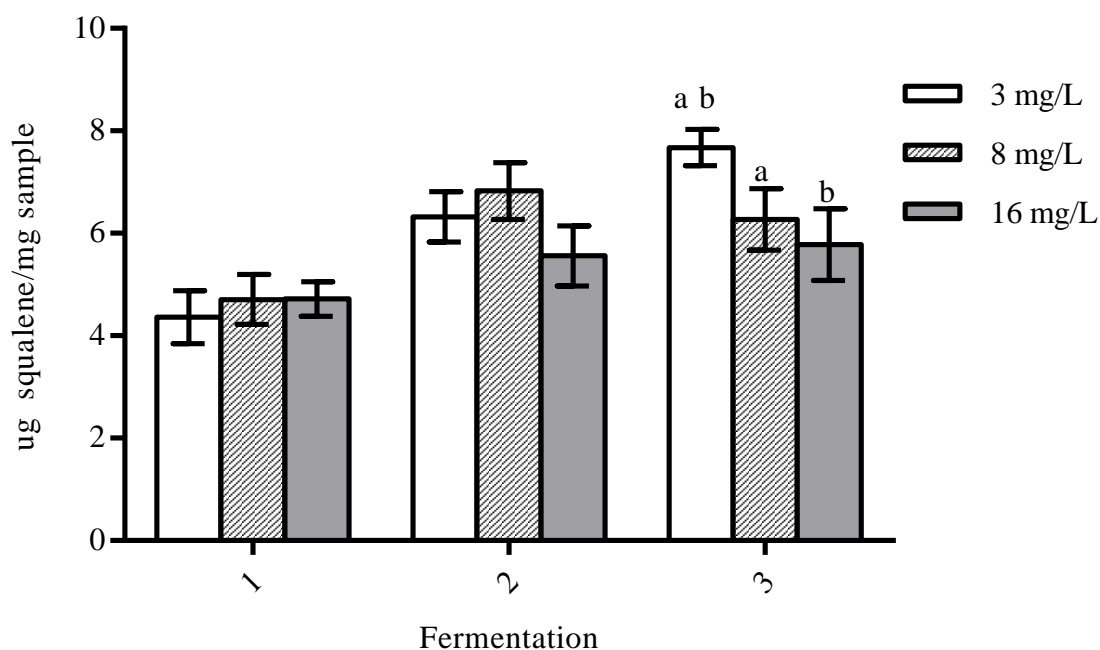
#### 8.3.4 Yeast Lipid Concentrations

The highest levels of ergosterol and zymosterol were found after propagation at concentrations of approximately 9  $\mu\text{g}/\text{mg}$  dry yeast and 3  $\mu\text{g}/\text{mg}$  dry yeast respectively (Table 8.3). The ergosterol and zymosterol levels were lower after fermentation at approximately 1  $\mu\text{g}/\text{mg}$  dry yeast and 0.09  $\mu\text{g}/\text{mg}$  dry yeast respectively.

The levels of squalene after the first fermentation for all treatments were approximately  $4.6 \pm 0.4$   $\mu\text{g}$  squalene/mg dry yeast for all DO concentration treatments (Figure 8.10). After the second fermentation, the concentrations of squalene increased in the cells to  $6.2 \pm 0.7$   $\mu\text{g}$  squalene/mg dry yeast for all DO concentration treatments. Finally, after the third fermentations, the concentrations of squalene in the cell were found to be significantly different between the different DO concentrations. The fermentation that started with 3 mg/L of DO contained  $7.7 \pm 0.4$   $\mu\text{g}$  squalene/mg dry yeast, 8 mg/L of DO contained  $6.3 \pm 0.6$   $\mu\text{g}$  squalene/mg dry yeast, and 16 mg/L of DO contained  $5.8 \pm 0.7$   $\mu\text{g}$  squalene/mg dry yeast (Figure 8.10).

**Table 8.3.** Levels of squalene, ergosterol, and zymosterol after yeast propagation (prop) and at hour 102 post primary fermentation for 3 re-pitched fermentations (1 – 3) in the yeast cells with the various levels of DO at the beginning of fermentation.

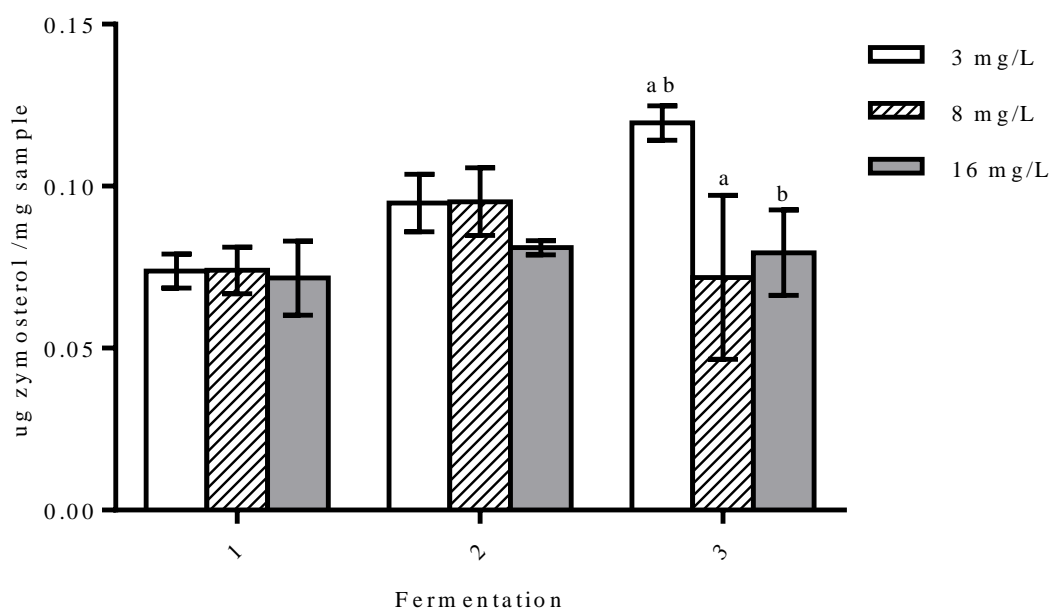
Compound	Ferm.	Average ( $\mu\text{g}/\text{mg}$ dry yeast) $\pm$ SD					
		3 mg/L DO		8 mg/L DO		16 mg/L DO	
Squalene	Prop	0.33	$\pm 0.059$	0.28	$\pm 0.074$	0.30	$\pm 0.038$
	1	4.36	$\pm 0.52$	4.71	$\pm 0.49$	4.72	$\pm 0.34$
	2	6.32	$\pm 0.49$	6.83	$\pm 0.55$	5.56	$\pm 0.59$
	3	7.67	$\pm 0.35$	6.27	$\pm 0.60$	5.78	$\pm 0.70$
Ergosterol	Prop	9.89	$\pm 1.64$	9.25	$\pm 0.38$	8.60	$\pm 0.76$
	1	1.73	$\pm 0.093$	1.68	$\pm 0.14$	1.69	$\pm 0.20$
	2	1.35	$\pm 0.15$	1.45	$\pm 0.015$	1.36	$\pm 0.091$
	3	1.33	$\pm 0.074$	1.44	$\pm 0.024$	1.46	$\pm 0.088$
Zymosterol	Prop	2.74	$\pm 0.39$	2.79	$\pm 0.56$	2.86	$\pm 0.62$
	1	0.074	$\pm 0.0053$	0.074	$\pm 0.0072$	0.072	$\pm 0.011$
	2	0.095	$\pm 0.0089$	0.095	$\pm 0.010$	0.081	$\pm 0.0022$
	3	0.12	$\pm 0.0053$	0.072	$\pm 0.025$	0.079	$\pm 0.013$



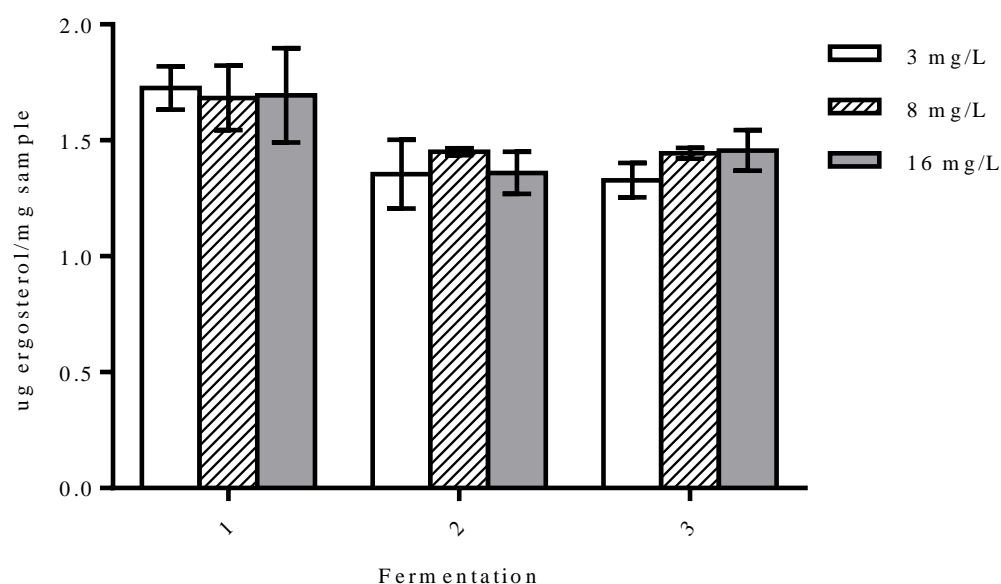
**Figure 8.10.** Levels of squalene in the cell post primary fermentation for three re-pitched fermentations with a designated initial DO level. The treatments that showed a significant difference by Tukey's test are marked by "a" or "b".

The two sterols investigated in this study to determine the influence of DO concentration were zymosterol and ergosterol. The levels of zymosterol increased from the end of the first fermentation to the end of the second fermentation from  $0.07 \pm 0.007$  to  $0.09 \pm 0.010$   $\mu\text{g}$  zymosterol /mg dry yeast (Figure 8.11). There were no significant differences between the levels of zymosterol in the cell and the different DO treatments for the first and second fermentation. For the third fermentation, the levels of zymosterol were significantly higher with  $0.120 \pm 0.005$   $\mu\text{g}$  zymosterol/mg dry yeast with the fermentation that contained 3 mg/L of DO when compared to the fermentations that contained 8 mg/L and 16 mg/L of DO which were  $0.072 \pm 0.025$  and  $0.079 \pm 0.013$   $\mu\text{g}$  zymosterol/mg dry yeast respectively.

The levels of ergosterol did not show any significant differences between the different DO treatments for each fermentation. The levels of ergosterol were highest after the first fermentation at  $1.7 \pm 0.1$   $\mu\text{g}$  ergosterol/mg dry yeast. After the second and third fermentations, the levels found were lower at  $1.4 \pm 0.1$   $\mu\text{g}$  ergosterol/mg dry yeast for both fermentations (Figure 8.12).



**Figure 8.11.** Levels of zymosterol in the cell post primary fermentation for three re-pitched fermentations with a designated initial DO level. The treatments that showed a significant difference by Tukey’s test are marked by “a” or “b”.



**Figure 8.12.** Levels of ergosterol in the cell post primary fermentation for three re-pitched fermentations with a designated initial DO level.

The other class of lipids analysed within the cell were saturated and unsaturated fatty acids. The concentrations of palmitic acid (16:0) were lowest after yeast propagation at  $7.1 \pm 1.4 \mu\text{g/mg}$  dry yeast. After the cells underwent fermentation, the concentrations of palmitic acid almost doubled to  $12.6 \pm 1.8 \mu\text{g/mg}$  dry yeast irrespective of the DO treatment (Table 8.4). The concentrations of stearic acid (18:0) stayed approximately the same between freshly propagated yeast and yeast after fermentation irrespective of the DO levels at  $3.6 \pm 0.2$  and  $3.1 \pm 0.4 \mu\text{g/mg}$  dry yeast respectively. The concentrations of palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) were highest in freshly propagated yeast at  $47.0 \pm 3.4 \mu\text{g/mg}$  dry yeast and  $28.4 \pm 2.1 \mu\text{g/mg}$  dry yeast. At the end of all the fermentations completed, the concentrations of 16:1n-7 and 18:1n-9 were both lower than the concentrations in freshly propagated yeast at  $12.6 \pm 1.8$  and  $3.8 \pm 1.2 \mu\text{g/mg}$  dry yeast respectively (Table 8.4).

All fatty acids analysed showed no significant difference between the DO treatments for all fermentations. However, with fatty acids palmitic acid (16:0) and palmitoleic acid (16:1n-7), the levels were significantly lower and higher in propagated yeast respectively than after the levels in all fermentation (see Appendix B).

**Table 8.4.** Major unsaturated and saturated fatty acids in the yeast cell after yeast propagation and at the end of fermentation (h 102) for three serial re-pitched fermentations.

Average (ug/mg dry yeast) $\pm$ SD					
Lipid	Ferm.	3 mg/L DO		8 mg/L DO	
		16 mg/L DO			
16:0	Prop	6.9	$\pm 1.4$	7.5	$\pm 2.3$
	1	12.0	$\pm 1.4$	11.5	$\pm 0.9$
	2	13.7	$\pm 2.2$	12.9	$\pm 1.8$
	3	14.1	$\pm 2.4$	12.0	$\pm 0.3$
16:1n-7	Prop	47.1	$\pm 3.0$	47.1	$\pm 7.0$
	1	13.9	$\pm 2.2$	12.8	$\pm 1.1$
	2	12.6	$\pm 2.5$	12.0	$\pm 1.1$
	3	12.0	$\pm 1.6$	11.8	$\pm 0.6$
18:0	Prop	3.6	$\pm 0.1$	3.5	$\pm 0.3$
	1	3.3	$\pm 0.3$	3.1	$\pm 0.2$
	2	3.2	$\pm 0.5$	3.0	$\pm 0.3$
	3	3.2	$\pm 0.5$	2.9	$\pm 0.1$
18:1n-9	Prop	28.5	$\pm 2.1$	28.1	$\pm 3.4$
	1	5.5	$\pm 0.8$	5.1	$\pm 0.6$
	2	3.2	$\pm 0.5$	3.1	$\pm 0.2$
	3	2.7	$\pm 0.4$	2.8	$\pm 0.1$

## 8.4 Discussion

### 8.4.1 Physical Fermentation

Both yeast in suspension and density attenuation models were demonstrated to show no significant differences between each of the DO treatments for each fermentation. It was hypothesised that with 3 mg/L of initial DO levels, the density attenuation rates would proceed slower than the fermentations with 8 and 16 mg/L of initial DO levels. A previously published study with yeast strain W34/70 showed that higher DO levels in the beginning of fermentation was related to higher growth in fermentation and fermentations that reach final density earlier than lower DO levels at the beginning of fermentation (Kucharczyk and Tuszyński, 2017). For this yeast strain, in the experimental study presented this hypothesis was not confirmed. The current work showed that the *Saccharomyces pastorianus* SMA yeast strain could complete fermentation with DO levels as low as 3 mg/L and with identical attenuation rates as fermentations with 8 mg/L and 16 mg/L DO. Most yeast strains require 8 – 12 mg/L DO (Gibson *et al.*, 2008b). However, it was determined that 3 mg/L of DO at the start of

fermentation was sufficient because there was substantial yeast growth observed at the start of fermentation. Also, the yeast in suspension trends with 3 mg/L dissolved oxygen were identical to the yeast in suspension trends in all re-pitched fermentations with 8 mg/L and 16 mg/L. It has already been established by Kirsop (1974) that one strain, NCYC 1236, grew during fermentation with only 2 ppm initial dissolved oxygen. Based on the results from the current study, it is hypothesised that strain SMA has a low requirement for oxygen similar to that observed for strain NCYC 1236.

In addition to this, a previously published study presented the idea that excess oxygen was detrimental to some yeast strains (Jakobsen and Thorne, 1980). The results from this study suggested that *S. pastorianus* SMA was not in the group of yeast strains where excess oxygen (i.e. 16 mg/L) is detrimental to the yeast growth. This was demonstrated by the yeast in suspension trends that were not significantly different for the fermentations with 3 mg/L, 8 mg/L and 16 mg/L dissolved oxygen. These results demonstrated that this yeast strain was robust, with the ability to perform within a wide range of dissolved oxygen concentrations. For this reason, strain SMA would be a good choice in a brewery that doesn't have equipment and instruments for maintaining consistent dissolved oxygen levels between fermentations.

#### **8.4.2 Beer Flavour Profile**

There was very little effect of the initial DO levels upon the levels of flavour compounds present by the end of fermentation. In the fermentation that was conducted with freshly propagated yeast, it was found that varying the DO treatment had no effect on any of the flavour compounds analysed. Kirsop (1974) suggested that freshly propagated yeast may carry an oxygen charge from propagation into fermentation and therefore not require oxygen during fermentation. The results in the study presented from the first fermentation agree with Kirsop's observation. Interestingly it was found that, only after the third fermentation, did the dissolved oxygen treatments have a significant effect on some flavour compounds that were produced. This suggested that when serial re-pitching with a yeast strain that had a low requirement for oxygen, an accumulated effect existed. The concentrations of 2,3-butanedione and propan-1-ol were found to be significantly higher with 16 mg/L initial DO levels compared to 3 mg/L initial DO levels after the third fermentation (Figure 8.9). A recent study by Kucharczyk and Tuszyński (2017) on an industrial scale showed that levels of propan-1-ol and 2,3-butanedione produced post fermentation using yeast strain W34/70 from initial dissolved oxygen levels in the wort of 7, 10, and 12 mg/L, were significantly



different. This observation aligns with the presented work, where propan-1-ol and 2,3-butanedione were significantly different depending on the DO treatment in the third fermentation. The strains in both studies were lager yeasts. The same, recently published, study found no significant difference between the esters produced and the varying levels of dissolved oxygen (Kucharczyk and Tuszyński, 2017). This was also consistent with the findings from the current study that the dissolved oxygen content had no impact on esters produced for each of the re-pitched fermentations.

This study also found differences in yeast growth and density attenuation rates between the initial DO treatments (Kucharczyk and Tuszyński, 2017). Since yeast growth differences were not observed in the current study, it is suggested that yeast strain W34/70 had a higher requirement of DO than the yeast strain, SMA, utilized in the current study

Lastly, the current study found no significant differences in the levels of acetaldehyde, whereas two previously published studies demonstrated that acetaldehyde levels are impacted by oxygenation levels (Maemura *et al.*, 1998; Kucharczyk and Tuszyński, 2017). Considering yeasts reduce acetaldehyde into ethanol (Pires and Brányik, 2015), and in the two published studies higher yeast growth correlated with higher levels of oxygen, it is hypothesised that the reduced acetaldehyde levels were connected with more yeast present. Seeing that the yeast growth was not impacted by the dissolved oxygen treatments in the current study, this could provide an explanation for the stable acetaldehyde levels observed between oxygen treatments.

#### **8.4.3 Yeast Lipid Concentrations**

Ergosterol and zymosterol concentrations in the yeast cell analysed were significantly higher, and squalene was significantly lower, in the propagated yeast than after fermentation (Table 8.3). Also, within the first and second fermentations, the sterols and squalene levels were not significantly affected by the dissolved oxygen treatments. Even when the yeasts were exposed to high levels of dissolved oxygen (16 mg/L) during fermentation, the cells did not maintain the high levels of sterols observed in the propagated yeast. This result suggested that the optimal concentrations of sterols maintained in the yeast cell between fermentation conditions versus propagation conditions, are different.

Previously published literature discussed that the function of sterols may include maintaining a proton gradient across the cell membrane (Haines, 2001). One other

previously published study found that the concentrations of calcium, magnesium, and zinc ions in the yeast cells were higher in the fermentation with freshly propagated yeast than the re-pitched fermentations (Aleksander *et al.*, 2009). Similarly, Deželak and colleagues found that levels of zinc in the fermenting medium were lowest in the fermentation medium with freshly propagated yeast when compared to 11 serial re-pitched fermentations (Deželak *et al.*, 2015a). A correlation may exist here with serial re-pitching that merits further investigation.

Furthermore, reflecting the trends found for the flavour compounds 2,3-butanedione and propan-1-ol, the dissolved oxygen treatments had an impact only on the third fermentation. Levels of squalene and zymosterol were significantly higher in the third fermentation with 3 mg/L of dissolved oxygen in the wort than the 8 mg/L and 16 mg/L treatments. It appeared that the effects of the dissolved oxygen treatments were only significant once the treatment was accumulated from three re-pitched fermentations. Interestingly, ergosterol, the major sterol in the yeast, was not affected by the oxygen treatments for all fermentations. Squalene is a substrate in all sterol biosynthesis. The fact that squalene was significantly different and ergosterol was not, suggests that the trace sterols may be affected by lower dissolved oxygen levels. The intermediate steps towards sterol biosynthesis after squalene utilise oxygen (Figure 8.1). The higher levels of squalene observed with 3 mg/L of dissolved oxygen compared to 8 and 16 mg/L treatments showed that the lower oxygen is hindering the synthesis of some sterols.

The levels of palmitic acid (16:0) were lowest and levels of palmitoleic acid (16:1n-7) were highest in propagated yeast. It is hypothesised that during propagation, high levels of palmitoleic acid were synthesized, and palmitic acid synthesis was not prioritized. Oxygen is necessary for forming the double bond in unsaturated fatty acids (Fornairon-Bonnefond *et al.*, 2002), such as palmitoleic acid, which was in excess during propagation. Conversely, when exposed to anaerobic conditions where oxygen is not in excess, the levels were reversed. Resulting in concentrations of palmitic acids that were higher, and palmitoleic acids that were lower.

These trends were not observed when considering the fatty acids, stearic acid (18:0) and oleic acid (18:1n-9). The concentrations of stearic acid in freshly propagated yeast were only significantly different from the concentrations of stearic acid found in the yeast after the third fermentation. The levels of stearic acid in the first and second fermentation were not significantly different from the levels found in the freshly

propagated yeast. Where the levels of palmitic and palmitoleic acid were dynamic when exposed to propagation conditions versus fermentation conditions, the levels of stearic acid remained conserved until the third fermentation. Interestingly, oleic acid was significantly different between propagated yeast, the first fermentation, and the second fermentation.

The levels of unsaturated fatty acids were lower at the end of fermentation. This could be assumed to be because unsaturated fatty acids require  $\frac{1}{2}$  O<sub>2</sub> molecule to form one the double bond for biosynthesis of an unsaturated fatty acid (Fornairon-Bonnefond *et al.*, 2002). Fermentation is an anaerobic process and at the beginning of fermentation oxygen is limited. Therefore, it makes sense that during propagation when the cells are exposed to excess oxygen, the levels of unsaturated fatty acids are higher than fermentation where oxygen is not as readily available.

Overall, the largest effect on fatty acid composition in the cell was between propagated yeast, and yeast after fermentation, not the re-pitched number or DO treatment. This provides insight into plausible reasons for the first fermentation in a brewery commonly being described as aberrant in the industry. During this period the yeast adjusts from propagation conditions to fermentation conditions. The change in palmitic acid, palmitoleic acid, and oleic acids were demonstrable physiological differences in the yeast between the two conditions. Additionally, researchers in this field know that glycogen levels change between propagated yeast and re-pitched yeast at the start of fermentations (Section 7.4.2). These are just two of many more physiological changes, still to be determined, that the cells undergo when transitioning between the two conditions.

## Chapter 9 – Conclusions and Future Work

‘Our imagination is the only limit to what we can hope to have in the future.’

-Charles F. Kettering

In the brewing industry, there are many reasons behind the rationale for the number of times a brewer will re-pitch yeast. In multinational breweries, the rationale tends to be company protocol. Many times, the rationale behind the re-pitched number limit for any brewery is from experience and observations made on past fermentations. In fact, there are a plethora of variables in brewing that are unique to a brand of beer. This may be the starting original density, temperature, dissolved oxygen content, FAN levels, mineral levels, and/or carbohydrate concentrations. These variables impose stresses onto the yeast and at different magnitudes. Each yeast strain is also unique in its own ability to adapt to these stresses. To address the commonly asked question, “how long should yeast be re-pitched?”, it depends.

### 9.1 Industrial Fermentations

Through analysis of industrial fermentations, the groundwork for this thesis was understood. Serial re-pitched fermentations at two breweries were monitored. One study monitored industrial serial re-pitched lager fermentations, while the second study monitored industrial serial re-pitched ale fermentations. Upon monitoring these fermentations, obstacles were discovered that impeded scientific analysis.

The malt bill for individual fermentations in the lager strain study was changed within the serial re-pitched process. Consequently, this may have influenced the results when investigating the yeast crop number between fermentations. Overall, however, the physical fermentation trends at the end of the re-pitching cycle did not significantly vary from physical fermentation trends at the beginning of the re-pitching cycle. Twelve flavour compounds were analysed in the produced beer and the results demonstrated that the levels of 2,3-butanedione, 2,3-pentanedione, and ethyl butyrate were significantly different dependent on the two different malt bills utilized. Only one of the twelve flavour compounds were significant related to the crop number, which was ethyl octanoate. Upon investigation of this trend, it may be that the trend was related to a longer yeast storage duration before starting the serial re-pitching cycle.

For the ale study, one yeast culture was used to re-pitch fermentations for different brands of beer each week. Once the fermentations reached completion, the yeast was collected and combined into one yeast storage tank. The collected yeast culture was re-pitched the following week into new fermentations for different brands of beer. This study monitored the re-pitched fermentations of one brand of beer in this process. This routine meant that portions of the yeast culture that was pitched into the monitored fermentation, had been previously exposed to different conditions. Two conditions of concern were the yeast storage times and previous exposure to fermentations with various wort compositions. Literature showed that these conditions effect the yeast (Rhymes and Smart, 2001; Bühligen *et al.*, 2013). The current study aimed to determine whether density attenuation rates were impacted by the crop number. Density attenuation trends between fermentations two, three (a), three (b), and nine did not significantly differ. For the other fermentations, the variations in the industrial brewing process likely contributed to the significant differences observed. This was assumed because the variations found between the density attenuation curves were not correlated with the crop number. To determine this, the midpoint of fermentation was calculated and correlated to the crop number. No significant correlations existed between the crop number and the fermentation midpoint. This demonstrated that the fermentability of the yeast was unchanged with the crop number and leaves the possibility that the yeast strain in these fermentations may be re-pitched longer. This finding was further strengthened with no significant linear correlations trends when considering the levels of flavour compounds analysed post fermentation with respect to the crop number apart from 3-methyl butanol, however, the levels reported were below flavour threshold.

While it was difficult to provide clear conclusions on the how the yeast crop affected the fermentation behaviour and beer quality in the two industrial studies, the obstacles observed were meaningful. In fact, developing an understanding of how the variables imposed by the brewer affect the serial re-pitching process would be valuable. By attempting to understanding these variables, brewers could make an informed decision to determine whether their decision could be detrimental to the re-pitching cycle.

## 9.2 Petite Mutation Frequencies

The first topic investigated was the impact of the presence of petite mutants in brewing, as levels of mutated cells have been reported to increase with serial re-pitching (Jenkins *et al.*, 2009). Yeast cultures were manipulated to contain various concentrations of petites from 3.66 % to 10.77 %. Fermentations were performed and the fermentation profile and flavour profiles produced were monitored. Interestingly from the varying levels of mutated cells in the experimental fermentations, the higher alcohols were not affected. This suggests that under or overproduction of higher alcohols by petites that was found in previous studies (Šilhánková *et al.*, 1970a; Morrison and Suggett, 1983; Ernandes *et al.*, 1993) were undetected at low concentrations of petites. The results also demonstrated that the concentration of esters and VDKs were positively correlated to an increase in petite mutations in the fermentation, with some esters having a stronger correlation than others. However, it is suggested that consumer detection of changes in flavour compounds between fermentation with varying levels of petites is unlikely.

The yeast in suspension trends were not significantly impacted by the low levels of petite mutations. This suggests that the overall flocculation of the culture used for fermentation was not impacted with low levels of petites present. The flocculation properties in published literature showed that there is an impact from petites at high levels (Ernandes *et al.*, 1993; Van Zandycke *et al.*, 2002). It was speculated that the difference in results between studies was due to different concentrations of petite mutations in the cultures. Previously published experiments argued that flocculation properties were only impacted when very high levels of petite mutated cells were present. This experiment, however, suggests that with the low levels of petite mutated cells common in industry, little impact on fermentation. Furthermore, the parameters in the density attenuation regression related to the midpoint, speed of fermentation, and final gravity of fermentation were unchanged between each frequency of RD yeast in the pitched yeast studied.

Considering all the parameters monitored during this current study, it is unlikely that low levels (< 10 %) of the petite mutated cells present in the pitched yeast are undetectable in the fermentation profile and final product for the yeast strain SMA.

## 9.3 Yeast Storage Duration

One key variable that was identified in industrial serial re-pitching operations was inconsistent storage times between re-pitched fermentations. The accumulated

effects of 18, 42, and 66 hour yeast storage times were investigated. Three series of re-pitched fermentations were completed in triplicate with the designated yeast storage time. The density trends were monitored for each of these fermentations. None of the yeast storage times were detrimental to the attenuation. All fermentations, regardless of the yeast storage duration, reached the final density. To investigate the impact of the storage times further, the consistency in reaching consistent target density attenuation trends for each storage time was observed. Visual observation of density attenuations and yeast in suspension measurements showed that longer yeast storage duration, the trends became less consistent. There were less instances where two yeast in suspension trends were not significantly different. This data showed that if the yeasts are exposed to longer yeast storage times, less consistent fermentations may be anticipated. At the same time, the larger range in variation within the 66 hour yeast storage times for density attenuation and yeast in suspension trends were not detrimental to the re-pitching process. All fermentations reached completion, and enough yeast was collected to re-pitch the subsequent fermentation.

The beer quality, however, is more important than slight variations in physical fermentation profiles. The beer quality is ultimately how the consumer judges a beer. Between these serial re-pitched fermentations, six of the 12 flavour compounds analysed were not significantly affected by the different yeast storage treatments. Those flavour compounds were propan-1-ol, acetaldehyde, acetone, ethyl acetate, isoamyl acetate, and ethyl butyrate. The other six flavour compounds: 2,3-butanedione, 2,3-pentanedione, ethyl hexanoate, isobutanol, 2 methyl butanol, and 3 methyl butanol were significantly affected by the yeast storage treatment. In all cases, the longest storage duration overall had higher levels of the flavour compound than the other two shorter storage durations. The change in the levels of these flavour compounds between each yeast storage time for each re-pitched fermentation was calculated. It was found that the change in flavour compound levels did not exceed the flavour threshold for the given flavour compound. Even though the yeast storage duration had a significant effect on those six flavour compound levels produced in fermentation, it is unlikely the change would be detected by a consumer. Therefore, yeast storage times within the re-pitched cycle varying from 18 hours to 66 hours would yield an acceptable fermentation profile with minimal changes in beer quality.

#### 9.4 Dissolved Oxygen Variation in Wort

Another variable that is controllable to the brewers, but is often not, is the dissolved oxygen level of the wort. Previously published research showed that the level of wort oxygenation impacted the levels of esters and higher alcohols produced (Verbelen *et al.*, 2009), the yeast growth during fermentation (David and Kirsop, 1973), and the speed of the fermentation (Kucharczyk and Tuszyński, 2017).

The study examined in this thesis investigated how the physical fermentation, beer flavour profile, and lipids in yeasts were affected by 3mg/ 8 mg/L, and 16mg/L of DO in the wort for three serial re-pitched fermentations. Interestingly, the density attenuation trends and yeast in suspension trends were the same between 3 mg/L, 8 mg/L, and 16 mg/L initial dissolved oxygen levels in the wort for three serial re-pitched fermentations. These results suggested that the yeast strain utilized had a low requirement for dissolved oxygen. The experimental dissolved oxygen levels were not lacking because yeast growth was observed during fermentation. Kucharczyk and Tuszyński (2017) found that with a strain that was impacted by their experimental levels of dissolved oxygen in wort, higher levels of dissolved oxygen were associated with more yeast growth in fermentation. Based on that published study, if dissolved oxygen was limiting, a difference in yeast in suspension trends would have been observed in this current study between 3, 8, and 16 mg/L of dissolved oxygen in the wort. This was not observed.

Small changes were observed related to the DO treatments in the flavour profiles and concentrations of some lipids for the third re-pitched fermentations. Levels of 2,3-butanedione and propan-1-ol were significantly different between the fermentations with 3 mg/L and 16 mg/L of dissolved oxygen. This showed that, while not originally apparent, there was an accumulated effect from the low (3 mg/L) and high (16 mg/L) of the DO treatment. David and Kirsop proposed that freshly propagated yeast “carry” some oxygen from the propagation step into the first fermentation (David and Kirsop, 1973). The results from the current study and the observation from David and Kirsop (1973) demonstrated that the effects of varying dissolved oxygen levels on fermentation may not always be immediately apparent.

Finally, the lipid profile of the yeast cells at the end of each fermentation were also analysed. The highest levels of sterols found in the yeast cells was immediately after yeast propagation. This was logical, because sterol biosynthesis requires oxygen and propagation delivers excess oxygen. During fermentation, regardless of the



dissolved oxygen level in the wort, the levels of sterols declined. In fact, the major sterol in yeast, ergosterol, decreased by approximately 9-fold by the end of the first fermentation regardless of the dissolved oxygen treatment. This suggested that yeast's quota for concentrations of sterols in the cell during fermentation conditions and propagation conditions, are different. Furthermore, the same accumulated effect observed for two flavour compounds, was also observed for squalene and zymosterol. Only after the third fermentation, the levels of squalene and zymosterol in the cell were significantly different between 3 mg/L and 16 mg/L dissolved oxygen treatments. This result and the correlating result with propan-1-ol and 2,3-butanedione levels in the beer, showed that changes exist on cellular levels that were not apparent in the physical fermentation data, with the modelling methods.

Considering previously published research and the experimental work presented in this thesis, researching the effect of serial re-pitching is a balancing act. Physiological, genomic, and metabolic changes take place on a cellular level during serial re-pitching, which may not also be apparent when monitoring the density attenuation or yeast growth and flocculation trends during fermentation. At the same time, not all changes on a cellular level experienced by serial re-pitching may impact the quality of the beer. Ultimately the final beer quality is the most important consideration for the commercial brewer.

## **9.5 Future Work**

The process of serial re-pitching has many complexities. Previously published literature has provided an insight into the challenges presented by the serial re-pitching process. Furthermore, one thesis does not have enough time, and resources to give the topic enough justice. There are many elements involved in serial re-pitching that deserve further investigation.

The petite mutation experiment provided a framework for the occurrence of petites at frequencies of their natural occurrence (Chapter 6). Petite mutations are a completely random mutation, which can affect portions of the mtDNA or the entire mtDNA. There are multiple variations of this study that could be performed. It could be valuable to determine whether the linear correlations observed with esters and the frequency of petite mutations in the pitched yeast would continue past the 10.77 % frequency studied. Furthermore, the scope of the study could be expanded to investigate naturally occurring petites. Individual petites could be isolated from breweries and cultured. If the same experimental design was performed multiple times, however by

culturing the petites from a different single petite colony each time, that could determine if petite mutations present inflict a universal effect.

Overall, out of all the results from this thesis, the effect that levels of DO in the wort at the beginning of fermentation have on yeast and fermentation, deserves more attention. A phenomenon observed with serial re-pitching is that propagated yeast accumulates higher levels of zinc, magnesium, and calcium in their biomass than observed with re-pitched yeast (Aleksander *et al.*, 2009). This thesis highlighted that between propagated yeast and re-pitched yeast, the yeast lipid compositions were different, and this observation was not related to the amount of dissolved oxygen exposed to the cells (Chapter 8). Future work should investigate the correlation between the sterol composition in the yeast cell membrane and the release of metal ions from the yeast cell during re-pitched fermentations. A published review mentioned that sterols in yeast may play a role in the proton gradient across the membrane (Haines, 2001). The lipid composition and metal concentration in the yeast cells could be an important correlation in understanding one of the many changes that take place during serial re-pitching.

## Appendix A

**Table A.1.** TTC Overlay Technique results from the yeast cultures at the beginning of the four fermentations completed.

Fermentation	Replicate	Total Petites	Total Cells Counted	% Petites	Average	SD
1	1	71	648	10.96	<b>10.66</b>	<b>1.22</b>
	2	57	645	8.84		
	3	66	680	9.71		
	4	65	635	10.24		
	5	52	576	9.03		
	6	58	546	10.62		
	7	79	652	12.12		
	8	81	650	12.46		
	9	61	549	11.11		
	10	63	548	11.50		
2	1	42	705	5.96	<b>5.10</b>	<b>0.73</b>
	2	44	736	5.98		
	3	39	711	5.49		
	4	34	707	4.81		
	5	39	766	5.09		
	6	36	801	4.49		
	7	41	748	5.48		
	8	36	645	5.58		
	9	26	624	4.17		
	10	26	664	3.92		
3	1	68	646	10.53	<b>8.39</b>	<b>1.10</b>
	2	46	593	7.76		
	3	41	614	6.68		
	4	51	603	8.46		
	5	45	621	7.25		
	6	51	600	8.50		
	7	65	686	9.48		
	8	63	706	8.92		
	9	52	621	8.37		
	10	51	639	7.98		
4	1	31	586	5.29	<b>3.66</b>	<b>1.04</b>
	2	27	588	4.59		
	3	17	596	2.85		
	4	16	534	3.00		
	5	29	622	4.66		
	6	18	620	2.90		
	7	26	561	4.63		
	8	11	454	2.42		
	9	14	536	2.61		
	10	18	490	3.67		

## Appendix B

**Table B.1.** Two-way ANOVA results from the saturated and unsaturated fatty acid concentrations at the end of three serial re-pitched fermentation and in freshly propagated yeast.

<b>Lipid</b>	<b>ANOVA table</b>	<b>SS</b>	<b>DF</b>	<b>MS</b>	<b>F (DFn, DFd)</b>	<b>P value</b>
18:1n-9	Interaction	0.3507	6	0.05845	F (6, 22) = 0.03995	P = 0.9997
	Row					
	Factor	3308	3	1103	F (3, 22) = 753.5	P < 0.0001
	Column					
	Factor	0.7536	2	0.3768	F (2, 22) = 0.2575	P = 0.7753
	Residual	32.19	22	1.463		
18:00	Interaction	0.2183	6	0.03638	F (6, 22) = 0.2819	P = 0.9394
	Row					
	Factor	1.277	3	0.4257	F (3, 22) = 3.298	P = 0.0394
	Column					
	Factor	0.2369	2	0.1185	F (2, 22) = 0.9179	P = 0.4141
	Residual	2.839	22	0.1291		
16:1n-7	Interaction	2.36	6	0.3934	F (6, 22) = 0.06497	P = 0.9987
	Row					
	Factor	6382	3	2127	F (3, 22) = 351.3	P < 0.0001
	Column					
	Factor	1.458	2	0.7291	F (2, 22) = 0.1204	P = 0.8871
	Residual	133.2	22	6.055		
16:00	Interaction	7.973	6	1.329	F (6, 22) = 0.4104	P = 0.8642
	Row					
	Factor	173	3	57.67	F (3, 22) = 17.81	P < 0.0001
	Column					
	Factor	3.206	2	1.603	F (2, 22) = 0.4950	P = 0.6162
	Residual	71.24	22	3.238		

**Table B.2.** Tukey's Multiple Comparisons Test to determine which values were significantly different.

<b>Lipid</b>	<b>Tukey's multiple comparisons test</b>	<b>Mean Diff.</b>	<b>95 % CI of diff.</b>	<b>Significant?</b>
18:1n-9	1 vs. 2	2.084	0.5009 to 3.668	**
	1 vs. 3	2.569	0.9855 to 4.152	***
	1 vs. prop	-23.08	-24.77 to -21.39	****
	2 vs. 3	0.4845	-1.099 to 2.068	ns
	2 vs. prop	-25.17	-26.86 to -23.47	****
	3 vs. prop	-25.65	-27.34 to -23.96	****
18:0	1 vs. 2	0.07301	-0.3973 to 0.5433	ns
	1 vs. 3	0.2587	-0.2116 to 0.7289	ns
	1 vs. prop	-0.315	-0.8177 to 0.1878	ns
	2 vs. 3	0.1857	-0.2846 to 0.6559	ns
	2 vs. prop	-0.388	-0.8907 to 0.1147	ns
	3 vs. prop	-0.5737	-1.076 to -0.07093	*
16:1n-7	1 vs. 2	0.8699	-2.351 to 4.091	ns
	1 vs. 3	1.663	-1.558 to 4.885	ns
	1 vs. prop	-33.52	-36.96 to -30.08	****
	2 vs. 3	0.7936	-2.428 to 4.015	ns
	2 vs. prop	-34.39	-37.83 to -30.95	****
	3 vs. prop	-35.18	-38.63 to -31.74	****
16:0	1 vs. 2	-1.549	-3.904 to 0.8065	ns
	1 vs. 3	-0.7976	-3.153 to 1.558	ns
	1 vs. prop	4.729	2.211 to 7.247	***
	2 vs. 3	0.7514	-1.604 to 3.107	ns
	2 vs. prop	6.278	3.760 to 8.796	****
	3 vs. prop	5.527	3.009 to 8.045	****

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